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Development of a VLP-based HCV vaccine candidate

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Mestrado em Biologia Molecular e Genética

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2016

Master's Thesis in Molecular Biology and Genetics, Faculdade de Ciências, Universidade de Lisboa, held at the Animal Cell Technology Unit from Instituto de Biologia Experimental e Tecnológica and Instituto de Tecnologia Química e Biológica, Universidade NOVA de Lisboa (IBET/ITQB-UNL) under the supervision of Doutora Ana Sofia Coroadinha.

i. Acknowledgements

I would like to acknowledge all the people directly or indirectly involved in this thesis.

To Dr. Paula Alves, for giving me the opportunity to do my master thesis at Animal Cell Technology Unit at ITQB NOVA/IBET, for the good working conditions offered and for being a strong example of leadership.

To Dr. Ana Sofia Coroadinha, for accepting to be my co-supervisor, for giving me the opportunity to join the Cell Line Development and Molecular Biotechnology Laboratory and for the supervision.

To Dr. Ana Rita Matos, for accepting to be my supervisor and for always being available to help during my master thesis work at FCUL.

To Hugo Soares, for his guidance, for the practical help and always being there to help me whenever I needed with patience throughout the time. For constant encouragement and always believing in my work. For helping me grow as a scientist.

To all the ACTU colleagues for the good working environment, friendship and the help during this year. A special thanks to 'virus lab' working people, for all the scientific discussions and encouragement during the good and the bad moments; to Daniel Mestre for sharing jokes with me.

Um especial 'thanks' às pessoas mais importantes da minha vida: os meus pais, o meu irmão e toda a família no geral, pelo amor e apoio incondicional, por acreditarem em mim e por estarem sempre presentes e proporcionarem-me sorrisos nos momentos menos bons.

À Sofia, à Íris, à Vanessa e ao Tiago, pela melhor companhia que alguma vez se podia ter na descoberta da vida académica, por todo o apoio e pela amizade resistente às distâncias.

Ao Aron pela dedicação incessante e paciência.

Ao Cubano, pela inspiração, compreensão e gargalhadas 'non-stop'.

Aos amigos da velha-guarda – Rhaisa, Carlos, Nice, Gonçalo, Max, Cássia, Rubinho, Grazielli, Joana e Felipe Premoli -, por se manterem como se fosse ontem.

Ao amável casal, Alexandre e Lúcia Kiala, pelo companheirismo, encorajamento e forte exemplo de liderança.

A todos, o*brigada!*

ii. Abstract

The Hepatitis C Virus (HCV) infects approximately 3% of the world population, being one of the major causes of liver cirrhosis and hepatocellular carcinoma. The development of safe, effective and affordable prophylactic and therapeutic vaccines against HCV has become an important medical priority; however, there are many obstacles to its development. In recent years, strategies of viral antigen delivery, as virus-like particles (VLPs), have been developed for use in vaccines and marketed, as Human Papillomavirus (HPV) vaccine. The main objective of this work consists in the development and production of HCV-like particles as HCV vaccine candidates.

We started by evaluating two different cell substrates – HEK293 and HuH-7 - for HCV-LPs production. HEK293 and HuH-7 cell lines were transduced with three cassettes constructed with a lentiviral backbone that code for HCV genes. The mRNA, as well as protein of all viral components was detected in transduced cells. Afterwards, HCV-LPs were purified using sucrose cushion ultracentrifugation. The impact of Fetal Bovine serum (FBS) reduction of concentration in HCV-LPs production was also analyzed. Our results indicate that HEK293 produced HCV-LPs are similar to those produced in HuH-7 cells with the advantage of their slightly higher production yields. Therefore, we proceed with the development of a stable cell line using defined gene expression loci, using a tagging and targeting strategy.

We started by tagging HEK293 cells with *pTagFRT_mCherry* expression cassette. After 72 hours under selective hygromycin pressure we obtained different cell populations with heterogeneous mCherry fluorescence intensity. These populations were independently subjected to limiting dilution to isolate homogeneous individual cell clones. 16 clones were analyzed for mCherry expression and the 10 clones showing higher mCherry fluorescence were screened by RT-qPCR for single-copy integration of tagged mCherry cassette. For those clones with single copy integration, we evaluated functionality of the genetic construct by assessing retroviral vector production. Clones #206 and #311 were chosen to produce HCV-LPs.

Clones #206 and #311 were transfected with the *pTarLoxP_E1E2*, a targeting vector expressing HCV envelope proteins. Puromycin resistant cells, indicative of Cre-mediated cassette exchange, were obtained from clone #311, that shown 7% of cassette exchange efficiency. A limiting dilution was performed to isolate targeted. Clone #311_F8 was chosen for expansion and Flipase cassette exchange. Thus, parental clone #311 and clone #311_F8 were transfected with the exchange vector *pTarFRT_Core*, antibiotic selection and clone expansion of resistant cells is still ongoing.

This work directly contributes to the development HCV-like (HCV-LP) particles that mimic HCV structure and antigen display thus contributing for the development of a vaccine against HCV.

Keywords: HCV, VLP, Lentiviral vectors, RMCE, Vaccines.

iii. Resumo

O Vírus da Hepatite C (HCV) infeta aproximadamente 3% da população mundial, sendo uma das principais causas de cirrose e carcinoma hepatocelular. O desenvolvimento de uma vacina efetiva e segura, profilática e terapêutica contra o HCV é urgente, de forma a controlar a epidemia global. No entanto, existem diversos obstáculos para o seu desenvolvimento, incluindo a elevada variabilidade do genoma do HCV, a falta de sistemas de cultura eficientes para a replicação do vírus infecioso e de modelos animais para estudar a replicação do HCV e a sua patogénese. Considerando estes obstáculos, nos anos mais recentes, estratégias de entrega de antigénios virais, como as partículas semelhantes a vírus (VLPs), têm sido desenvolvidas para o uso em vacinas, como é o caso da vacina contra o Vírus do Papiloma Humano (HPV).

O principal objetivo deste projeto é o desenvolvimento e produção de HCV-LPs como candidatas a uma vacina contra o HCV.

Uma linha celular estável pode ser usada como produtora de VLPs, contudo, o seu desenvolvimento é significativamente moroso e laborioso. Além disso, e independentemente do sistema de expressão, quando se pretende expressar biofármacos multiméricos, como as VLPs, o rendimento é geralmente muito baixo.

De forma a superar estas limitações, para o projeto foram estabelecidos dois objetivos: a avaliação de duas linhas celulares diferentes (HEK293 e HuH-7) transduzidas com vetores lentivirais com os genes do HCV necessários para produção de HCV-LPs e, posteriormente, o desenvolvimento de uma linha celular estável derivada de HEK293, usando a tecnologia de troca de cassete mediada por uma recombinase (RMCE).

Os vetores lentivirais têm sido utilizados como uma ferramenta de terapia génica uma vez que são capazes de transferir um grande inserto exógeno para as células-alvo, podendo ser utilizados como vetores para o tratamento de diversas patologias, como por exemplo, doenças neurológicas e síndrome de imunodeficiência adquirida (SIDA); possuem uma baixa imunogenicidade e são capazes de transformar geneticamente as células em não divisão como os hepatócitos, ao contrário de outros tipos de vetores virais. A produção de vetores lentivirais é realizada em células de mamíferos, geralmente, com origem humana ou de ratinho, e requer a utilização de quatro cassetes de expressão independentes, no caso do sistema de terceira geração, que codificam *gag-pro-pol* (proteínas virais estruturais e enzimáticas), *rev* (proteína auxiliadora), *env* (glicoproteínas do envelope) e o transgene, que contém o gene de interesse clonado no esqueleto do genoma viral. Cada estágio de inserção requer a seleção por antibiótico, de acordo com a marca de seleção incluída na cassete com o transgene.

Por outro lado, os sistemas de RMCE têm sido utilizados para introduzir cassetes de expressão em regiões pré-definidas do genoma celular por intermédio de uma reação de recombinação homóloga de recombinases, que envolve dois passos. O primeiro consiste na introdução de uma cassete "tagging" flanqueada por duas sequências RT (do inglês, recombination target sites) heteroespecíficas incompatíveis no genoma celular. Este processo de integração no genoma celular ocorre de uma forma aleatória. O segundo consiste na troca da cassete por uma cassette "targeting" flanqueada pelas mesmas sequências RT heteroespecíficas e este processo é mediado por uma recombinase.

Como dito anteriormente, o trabalho desenvolvido neste projeto começou por centrar-se na avaliação de duas linhas – HEK293 e HuH-7 – para produção de HCV-LPs. Para tal, foram contruídos cinco vetores lentivirais no sentido de expressarem os genes do HCV (*Core, E1, E2, p7, NS2, NS3, NS4a, NS4b* e *NS5a*). As células foram transduzidas sequencialmente por três destes vetores e selecionadas, respetivamente, consoante a marca de seleção presente na cassete com o transgene. Posteriormente à seleção, analisou-se a expressão dos genes do HCV e das respetivas proteínas das células transduzidas e verificou-se que todos os componentes virais estavam a ser expressos. No entanto, a deteção da expressão das proteínas p7 e NS2 não foi possível uma vez que não há anticorpos disponíveis comercialmente para a estirpe de HCV em estudo.

Uma vez confirmado que as proteínas estruturais estavam a ser expressas e, portanto, cumpridos os requisitos essenciais para se obterem HCV-LPs, avaliou-se a produção de HCV-LPs. Então, recorreu-se ao método de centrifugação da "cama" de sucrose para se purificarem as psedopartículas. Tendo em conta os rendimentos de HCV-LPs obtidos pós-purificação, as linhas celulares HEK293 parecem ser um bom substrato celular para a sua produção.

Um estudo sobre a influência de soro fetal bovino (FBS) na produção de HCV-LP também foi realizado, em colaboração com o Downstream Process Development Laboratory, e verificou-se que, de forma geral, a produção de HCV-LPs diminuiu para HEK293 e HuH-7, quando as concentrações de soro foram reduzidas.

O segundo objetivo desta tese consistiu no desenvolvimento de uma linha celular estável produtora de HCV-LPs. Para o funcionamento do sistema RMCE foram necessárias duas cassetes distintas e complementares ("tagging"/"targeting"). As cassetes tagging *pTagLoxP_GFPZeo* e *pTagFRT_mCherry* expressavam os genes repórtes *GFP* e *mCherry*, respetivamente. Por outro lado, as cassetes targeting *pTarLoxP_E1E2* e *pTarFRT_Core* expressavam, respetivamente, os genes *E1 e E2* e *Core* do HCV. Em ambas as situações, a expressão da marca de seleção foi necessária para garantir a expressão estável das cassetes no genoma da célula. Enquanto a integração da cassete tagging no genoma se dá através de um processo aleatório, a integração da cassete targeting é o resultado da troca eficiente por recombinação de locais específicos mediada por uma recombinase.

Os clones #1, #2 e #3 de HEK293 que apresentavam previamente a cassete tagging *pTagLoxP_GFPZeo* foram transfetados com uma segunda cassete tagging *pTagFRT_mCherry* e colocadas em seleção com higromicina. As três populações apresentaram distribuições de fluorescência diferentes. De forma a obterem-se células homogéneas com níveis de expressão de *mCherry* semelhantes, fez-se um *limiting dilution* para se isolarem clones. Obtiveram-se 16 clones, que foram, posteriormente, analisados quanto à percentagem do número de células mCherry positivas e apenas 10 apresentaram elevada percentagem. Estes clones foram analisados por citometria de fluxo para comparar o nível de expressão de *mCherry*.

Antes do processo da troca da cassete, é importante que apenas uma cópia da cassete tagging seja integrada no genoma da célula para que o sistema RMCE seja funcional. Portanto, foi implementado um protocolo de RT-qPCR para analisar o número de cópias integradas da cassete tagging em cada clone relativamente a um controlo com uma única cópia. Considerando valores entre 0.6 e 1.4, como descrito por Bodin *et al* (2005), 6 dos 10 clones analisados mostraram a integração de uma única cópia. Posteriormente, desses clones foram escolhidos 4 (#103, #206, #301 e #311) para serem analisados quanto ao sinal de empacotamento (Ψ) colocado na cassette e os clones #206 e #311 foram selecionados para se proceder a recombinação homóloga.

Estes dois clones foram assim co-transfetados com a cassete target *pTarLoxP_E1E2* e com o plasmídeo que expressa a enzima recombinase Cre pelos métodos de transfeção polietilenimina (PEI) e fosfato de cálcio ($CaPO_4$). As células resistentes à puromicina, sugerindo o sucesso da troca da cassette, foram obtidas, mas apenas do clone #311, que revelaram por citometria de fluxo uma eficiência de troca de 7% para ambos métodos de transfeção. Estas células foram isoladas, obtendo-se 1 clone (#311_F8) com sinal *mCherry* positivo e *GFP* negativo. Este clone foi expandido e, posteriormente, foi co-transfectado com a segunda cassete target *pTarFRT_Core* utilizando novamente os métodos de PEI e $CaPO_4$. Neste momento, as células encontram-se sob seleção com neomicina.

Este trabalho contribuiu diretamente para o desenvolvimento de partículas HCV-LPs que mimetizam a estrutura do HCV e a exposição do antigénio e contribui assim para o desenvolvimento de uma vacina contra o HCV.

Palavras-chave: HCV, VLP, Vetores lentivirais, RMCE, Vacinas.

iv. Preface

This master thesis is within the scope of the project PTDC/EBB-BIO/102649/2008; entitled "Retroviral like particles: Improving potential as candidates vaccines for Hepatitis C" funded by the Portuguese Fundação para a Ciência e Tecnologia (FCT).

The main objective of this work consisted on the development and production of HCV-LP as HCV vaccine candidates, using a lentiviral transduction method to evaluate different cell substrates for HCV-LP production and, posteriorly, a RMCE method to develop a stable cell line for HCV-LP production.

Most the results described in this thesis were presented at one meeting international scientific meetings:

Rodrigues AF, Soares HR, Almeida AI, **Ferreira-Fernandes M**, Alves PM, Carrondo MJT, Coroadinha AS. *Development of HCV vaccine candidates using retroVLPs as manufacturing platform*. *10th Vaccine Congress 2016*, Amsterdam, the Netherlands.

Soares HR, Castro R, **Ferreira-Fernandes M**, Alves PM, Carrondo MJT, Coroadinha AS. *Development of an integrated process for an HCV vaccine candidate production*. *10th Vaccine Congress 2016*, Amsterdam, the Netherlands.

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viii. Abbreviations list

ATCC	American type culture collection
Blast	Blasticidin
bp	Base pair
CLDN1	Claudin-1
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EMCV-IRES	Encephalomyocaerditis virus-internal ribosome entry site
env	Envelope
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
Flp	Flipase
FRT	Flipase recombinase target
GAG	Glycosylaminoglycans
gag	Group-specific antigen
GOI	Gene of interest
HCV	Hepatitis C virus
HEK293	Human embryonic kidney 293 cell line
HEK293T	HEK293 derived cell line expressing large T antigen from SV40
Hygro	Hygromycin
HuH-7	Human hepatoma 7 cell line
IRES	Internal ribosome entry site
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LoxP	Cre recombinase target
LTR	Long-terminal repeat
LV	Lentiviral vector
MLV	Murine leukemia virus
MVB	Multivesicular bodies
Neo	Neomycin
OCLN	Occludin
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pol	Polymerase
PPT	Polypurine tract
pro	Protease
Puro	Puromycin
RMCE	Recombinase-mediated cassette exchange
RPLL22	Ribosomal protein L22
RRE	Rev responsive element
RSV	Rous sarcoma virus

RT-qPCR	Real-time quantitative polymerase chain reaction
SEC	Size-exclusion chromatography
SIN	Self-inactivating vectors
SR-BI	Scavenger receptor class B type 1
SSR	Site-specific recombinase
SUV	Subunit vaccine
SV40	Simian virus 40
TAE	Tris-Acetate-EDTA
TBS	Tris-buffered saline
T-flask	Tissue-culture flask
UTR	Untranslated region
VLP	Virus-like particle
VSV-G	Vesicular stomatitis virus G protein
WPRE	Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element
WT	Wild-type
Zeo	Zeocin

1. Introduction

Hepatitis C virus (HCV) was discovered in 1989 as the major causative agent of parental non-A, non-B hepatitis. HCV belongs to the *Flaviviridae* family[1] and has been classified in 6 genotypes and numerous subtypes[2,3]. Genotype 1 accounts for the majority of HCV infections worldwide, and subtypes 1a and 1b are predominant[3]. HCV is transmitted exclusively through direct blood-to-blood contacts between humans[4,5].

Approximately 170 million people are infected with HCV[1,4], which represents nearly 3% of the world population[6,7]. According to World Health Organization more than 350,000 deaths/year are due to HCV-related liver diseases[3]. The high prevalence of HCV is due to its subclinical acute infection which leads to chronic hepatitis in up to 80% of the cases[8,9].

HCV is implicated in liver steatosis, fibrosis, liver cirrhosis, hepatocellular carcinoma, type II cryoglobulinemia and non-Hodgkin's lymphoma[8,9], and is the primary reason for liver transplantations among adults in industrialized countries[10]. The variable effects of chronic infection among the individuals are due to the difference of their age, gender, immunity level and environmental healthcare[8]. Although the liver is major site of HCV replication, HCV RNA was also found in extra-hepatic cells and tissues such as peripheral blood lymphocytes, epithelial cells in the gut and in the central nervous system[11].

Standard therapy is based on the combination of non-HCV specific polyethylene glycol conjugated interferon-α with ribavirin. However, this treatment is relative toxic and its efficacy is limited, being effective in only approximately 50% of treated patients[12,13]. Recently, the standard treatment has been supplemented with NS3/NS4a protease inhibitors and NS5B polymerase inhibitors, particularly in patients infected with genotype 1[12,14] which are the most resistant to standard therapy[3].

At present time, there is no universal, highly effective therapy to chronic HCV infection. Therefore, the development of safe and effective vaccines against HCV is an important priority to control the global epidemic[14,15].

1.1. Hepatitis C virus

1.1.1. HCV genome and proteins

HCV is an enveloped virus with positive single-stranded RNA genome and its genome comprises a single open reading frame (ORF) of about 9.6 kb in length that is flanked by two untranslated regions (UTRs) – 5´UTR and 3'UTR[16]. The 5'UTR contains an internal ribosome-entry site (IRES)[16] that mediates the initiation of viral-RNA translation in a cap-independent manner while

3'UTR is crucial for efficient RNA replication[6,10]. The ORF encodes a large polyprotein precursor that is cleaved by cellular and viral proteases generating 10 different viral proteins[1,10].

HCV structural proteins Core, E1 and E2 are released after cleavage of polyprotein by host endoplasmic reticulum (ER) signal peptidase and signal peptide peptidases, whereas the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B are released after the enzymatic cleavage of HCV polyprotein by viral proteases NS2/3 and NS3/4A[6,8] (Figure 1.1).

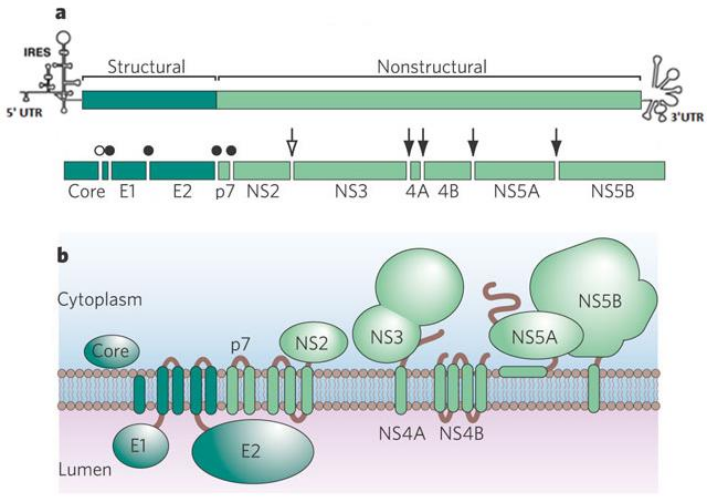


Figure 1.1. **HCV genome organization and polyprotein processing. A.** The structure of the viral genome, including the open reading frame encoding structural and nonstructural genes, and 5' and 3' UTRs. Open circle refers to C-terminal cleavage of Core by signal peptide peptidase; closed circles indicate the signal peptidase cleavage sites; open arrow indicates autocatalytic cleavage of the NS2-NS3 junction; and black arrows refer NS3/4a proteinase complex cleavage sites. **B.** The HCV proteins with respect to a cellular membrane. (Adapted from [11]).

The core protein is further processed by a signal peptide peptidase into a mature protein of 21 kDa. Mature core protein interacts with viral RNA and forms the nucleocapsid[6,10,17]. E1 and E2 are viral envelope glycoproteins with 35 kDa and 72 kDa[8], respectively. Both envelope proteins are type I membrane proteins responsible for receptor binding and entry into target cells[8,10,17]. p7 protein is a small (63 amino acids) hydrophobic peptide of only 7 kDa, which functions as an ion channel after oligomerized[5,6]. NS2 protein has 23 kDa[6] and, together with the N-terminal protease domain of the NS3 protein, forms a catalytically active protease that mediates NS2/3 cleavage[18]. NS3 protein has 69 kDa and has two distinct enzymatic activities: the N-terminal part of the NS3 protein is a serine-protease mediating the cleavage of NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B. The C-terminal part of the NS3 protein contains an NTPase and an RNA-helicase important for viral genome replication[6,18]. For NS3 to function as protease it needs NS4A as co-factor which is a small 6 kDa protein[18]. NS4B is a very hydrophobic 27 kDa protein[6]. NS4B induces membrane rearrangement with consequent formation of a membranous structure where viral RNA replication and assembly occurs[10,18]. NS5A is a membrane-anchored phosphoprotein with 56 kDa[19] that regulates viral replication through its

interaction with NS5B[6,10]. Finally, NS5B protein has 68 kDa, it is an RNA-dependent RNA polymerase (RdRp), that catalyses the replication of HCV RNA[6,18].

1.1.2. HCV life cycle

Attachment and entry of HCV to the host cell are the first steps in the virus life cycle[19]. Virus entry is a result of a fusion process between viral and cellular membranes[4]. Membrane fusion is mediated by specific interactions between viral surface glycoproteins (E1 and E2) and host cell receptors (CD81, SR-B1, LDLR, Occludin, Claudin-1)[6,8,20]. This interaction triggers decrease in pH of the endocytic compartment, leading to membrane fusion and nucleocapsid release to the cytosol[6,20]. Cell-associated attachment molecules, such as the low-density lipoproteins (LDLs) and glycosaminoglycans (GAGs), are involved in early steps of the process facilitating the described interactions between viral glycoproteins and cellular receptors[6,20,21].

After decapsidation, the positive-strand HCV RNA genome is delivered to the cytoplasm. This RNA molecule is translated by cellular ribosomes, and the nascent polyprotein is translocated to and processed in the endoplasmic reticulum (ER)[10]. By definition, viral structural proteins form the viral particle while viral nonstructural proteins form the replicase complex[5]. The replicase complex initiates the synthesis of intermediate negative-strand RNA, which is used as template for the generation of new positive-strand RNA molecules which are encapsidated in a newly formed virion[5,10]. Virus production occurs by virion budding into the lumen of the ER which results in the formation of multivesicular bodies (MVB)[10,22]. Finally, infectious virions are released from infected cells by fusion of MVB with host cell cytoplasmic membrane[10,22] (Figure 1.2).

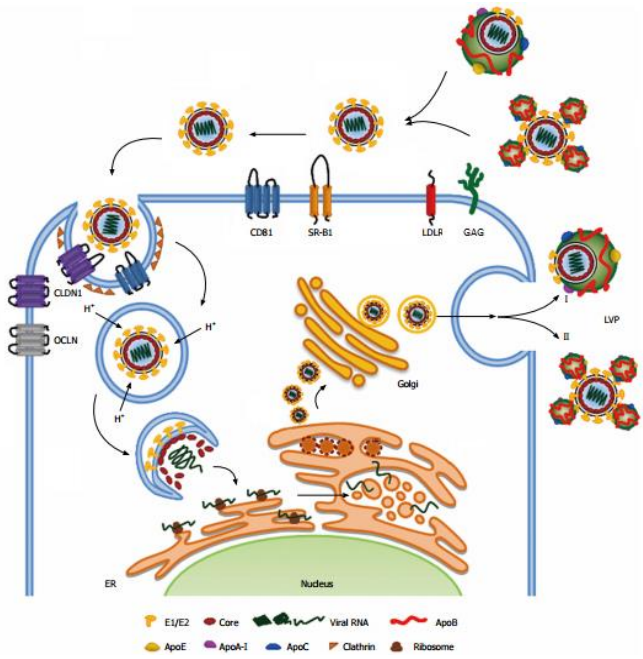


Figure 1.2. Hepatitis C virus life cycle. HCV first attaches itself to the host cell surface by interactions with GAG and/ or the LDLR. On the cell surface, virus is ables to interact with entry receptors such as CD81 and SR-BI. The virus-receptor complex

then translocates to the tight junctions where CLDN1 and OCLN act as cofactors and induce endocytosis. The virus-containing endosome is acidified in the cytoplasm and HCV RNA is released into the cytosol and translated in the ER, giving rise to a polyprotein that is cleaved into mature viral proteins. HCV RNA replication occurs and assembly into new virions starts in the ER. The viral envelope is acquired by budding into the ER. HCV particles are thought to be released via the constitutive secretory pathway in association with lipoviroparticles (LVP). This lipidation occurs either during budding (model I) or during egress via interaction between the virion and lipoproteins (model II). (Adapted from [20]).

1.2. Vaccines

Vaccination remains the most effective method for controlling and preventing infectious diseases[23,24]. Most vaccines currently available are based on inactivated or live attenuated pathogens. Even though these vaccines are highly effective, they present several limitations[24]. For instance, attenuated pathogens used in attenuated vaccines can revert to their pathogenic status as observed with oral Polio vaccine. Therefore, this strategy is not recommended to those viruses able to establish chronic infectious. Altogether the need to develop safer vaccines strategies urges[24,25].

Advances in recombinant DNA technologies and genetic engineering have contributed to the development of subunit vaccines (SUVs). SUVs are vaccines based on isolated components of the pathogen. Therefore, SUVs are considered safer than full pathogen-based inactivated or live attenuated vaccines. However, immunogenicity of SUVs is generally lower when compared to that of full pathogens, thus requiring higher doses, booster administrations or co-administration of adjuvants. Virus-like particles (VLPs) are a special subset of SUVs with enhanced immunogenicity[24].

1.2.1. Virus-like particles

Virus-like particles (VLPs) are multimeric protein complexes mimicking the morphology of native viruses but totally or partially lacking the viral genome[14,26,27]. VLPs are of small size, between 20 and 200 nm in diameter, allowing entry into the lymphatic vessels and passive drainage to the region of lymph nodes, and optimal uptake by professional antigen presenting cells (dendritic cells, B-lymphocytes and macrophages)[28,29].

These stable and versatile nanoparticles display excellent adjuvant properties being highly immunogenic *per se*[24,30]. They present high-density B-cell epitopes for antibody production and T-lymphocyte stimulation. This feature of VLP vaccines is likely to be a major contribution to their increased effectiveness[30,31].

VLPs have been used as useful platforms for delivering heterologous virus-derived antigens to the immune system and have come into focus for their diverse applications in vaccination, targeted drug delivery, gene therapy and immune therapy[14,25,29].

A VLP-based product candidate is hardly competitive in the market unless its manufacturing process is scalable and cost-effective. Therefore, strong product development efforts as modern molecular biology technologies are required. The design and development of new technologies and

platforms for the production of a whole new variety of VLPs and expression system is currently a hot-topic in vaccinology[25,26].

1.2.2. HCV vaccines

The development of safe, effective and affordable prophylactic and therapeutic vaccines against HCV has become an important medical priority; however, there are many obstacles to its development[14,15]. These limitations include the high sequence variability of HCV genome which is associated to the natural high-error prone of HCV RNA-dependent-RNA-polymerase[8,9], leading to the emergence of mutants resistant immune defenses and therapeutics; and the lack of culture system for virus replication and small-animal models for HCV replication and pathogenesis [10,14].

In recent years, several different strategies of HCV antigen delivery have been investigated for use in vaccines against HCV, with different degrees of success, these include the use of recombinant proteins, virus-like particles (VLPs), recombinant nonpathogenic live vectors and prime-boost approaches[14].

In parallel, due to their applicability in gene therapy, many efforts were invested to develop a variety of tools and protocols for industrial production and purification of viral vectors derived from retrovirus. Pseudotyped retroVLPs have been extensively used as scaffolds for candidate vaccines against HIV, HCV, HPV and many other virus[14,32]. In case of HCV, retroVLPs have been formed by the overexpression of the E1 and E2 envelope proteins together with murine leukemia virus (MLV), to generate chimeric viral particles[25,33]. These particles display E1 and E2 envelope proteins in the correct conformation and maintain a preferential tropism for hepatic cells therefore are commonly used to investigate the early events of HCV infection worldwide[14,25].

Several preclinical trials involving VLP-based vaccine candidates are currently underway and have demonstrated highly promising results in phase I, II and II clinical trials[14,32].

1.3. Mammalian cells engineering tools

1.3.1. Lentiviral transduction

Lentivirus belong to the *Retroviridae* family and can be used as a tool to deliver genetic material into mammalian cells[34,35].

The retrovirus genome encodes four genes: *gag* (group specific antigen), *pro* (protease), *pol* (polymerase) and *env* (envelope). The *gag* gene encodes the three main structural proteins: matrix, capsid and nucleocapsid. The *pro* gene encodes a protease responsible for cleaving Gag and Gag-Pol during virus maturation. The *pol* gene encodes two enzymes, the reverse transcriptase and the integrase, that catalyze the reverse transcription of the viral genome from RNA to DNA during the infection process and integrate the proviral DNA into the host cell genome, respectively. The *env* sequence encodes for both surface and transmembrane subunits of the envelope glycoprotein[34,36,37].

Additionally to the coding sequences, the retroviral genome presents cis-acting sequences that include two long terminal repeats (LTRs), a packaging signal (Ψ), and a polypurine tract (PPT). The LTRs are regulatory sequences of genome found at both the 5´and 3´ends that contain elements required to drive gene expression, reverse transcription and integration into the host cell chromosome. The packaging signal is required for specific packaging of the viral RNA into newly forming virions. The PPT functions as the site for initiating the positive strand DNA synthesis during reverse transcription[36-38].

Lentivirus, additionally to *gag*, *pro*, *pol* and *env* genes, have accessory genes (Figure 1.3), such as *tat*, *vpu*, *vif*, *nef* and *rev*. *Rev* gene codes for a protein which interacts with rev responsive element (RRE), a cis-acting sequence located in the middle of *env* gene allowing messenger RNA to be exported from nucleus intact and it is therefore the only accessory gene required for lentiviral vectors production[36,38,39].

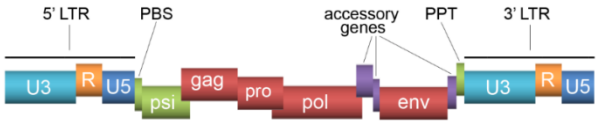


Figure 1.3. Schematic representation of the lentiviral genome (Adapted from [40]).

The capability to transduce non-dividing and dividing cells as well as the wide tropism afforded them by pseudotyping has enabled them becoming a highly efficient tool in therapy[34-36,41].

Four generations of lentiviral vectors (LVs) are currently considered. The vectors of third generation presented a higher level of biosafety and, currently, are the most commonly applied for the production of lentivirus[42]. These vectors require the transfection of four independent expression cassettes encoding *gag-pro-pol*, *env*, *rev* and transgene functions. *Gag-pro-pol* genes are expressed from a Cytomegalovirus (CMV) promoter and none of the accessory or regulatory proteins is present in this construct. Only rev accessory gene is maintained but is provided by a non-overlapping plasmid. Vector cassette for transgene expression is driven by a heterologous promoter, as virus LTRs were partially deleted[36,39,42].

1.3.2. Recombinase-mediated cassette exchange

Recombinase-mediated cassette exchange (RMCE) is a strategy which enables predictable expression of the gene of interest (GOI) at a pre-characterized genomic locus[43]. RMCE is a two-step procedure. First, a tagging cassette flanked by two heterospecific recombination target sites, a wild-type (wt) and a mutant target sequence, is random integrated in the genome. Second, a targeting cassette is integrated and the recognition sites for a site-specific recombinase (SSR) of tagging cassette are targeted to the locus of interest by homologous recombination. The SSR inserts a

replacement sequence into this tagged site[44,45]. Recombinase exchanges the two DNA regions flanked while maintaining the orientation of the regions[46,47]. For genomic RMCE, the target resides in the host genome, whereas the gene replacement cassette is introduced into the host cell, traditionally as part of a "donor plasmid"[44,48].

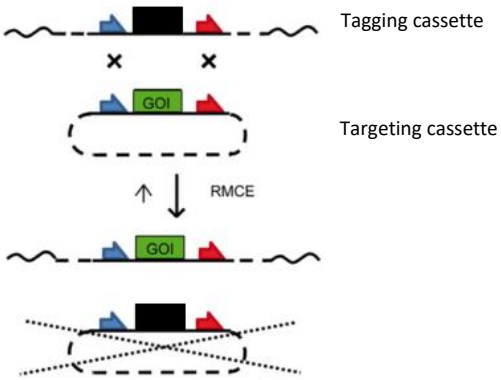


Figure 1.4. Recombinase-mediated cassette exchange. Half arrows mark target sites. These sites flank both the tag and the incoming cassette, which is provided as part of a donor plasmid. In the absence of this donor plasmid, the target cassette remains unperturbed. Upon its addition, a double-reciprocal cross-over between identical FRTs on each of the reaction partners is initiated by recombinases (Adapted from [49]).

The use of site-specific recombination systems, like the Flp/FRT (flipase/ flipase-recognition target sites) system that was originally isolated from the yeast *Saccharomyces cerevisiae* and Cre/*lox* from the bacteriophage P1 in mammalian cells allow integration of genes of interest into adequate genomic loci[50,51]. Differences between these systems go back to the particularities of these tyrosine recombinases regarding the details of the enzymatic mechanism and structure of their target sites[49].

1.3.2.1. Cre-RMCE

The CRE-*lox* site-specific recombination system has been applied to a variety of genetic systems. Cre recombinase, a 38.5 kDa protein, catalyzes reciprocal site-specific recombination between DNA elements termed *lox* sites. The *lox* site, *loxP*, is a 34 bp element composed of two 13 bp inverted repeats separated by an asymmetric 8 bp spacer region. Each 13 bp inverted repeat serves as a binding site for the recombinase, whereas the 8 bp spacer region participates in strand exchange during the recombination reaction. Because a productive recombination event requires synapsis of *lox* sites between their spacer regions, it is asymmetry of the spacer region that confers directionality to the recombination reaction[44,48,52].

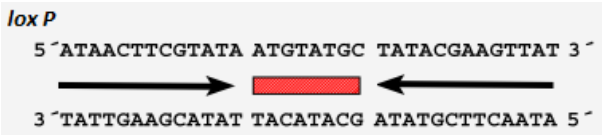


Figure 1.5. Architecture of wild-type lox sites. The arrows bracket the 13 bp palindromic arms that serve as binding sites for the Cre recombinase. The asymmetric 8 bp spacer (red box) is the region where breaking and rejoining events occur. (Adapted from [44]).

1.3.2.2. Flipase (Flp)-RMCE

The Flp/FRT system is similar to the Cre/lox system. Flp recombinase consists of a 13 kDa NH2-terminal domain and a larger 32 kDa COOH-terminal domain with the major determinants for DNA binding[44]. FRT site consists of genuine 48 bp, including three individual 13 bp Flp-recombinase binding elements, two of which (designated "a,a") form an inverted repeat around an 8 bp spacer. On one side of this core, the repeat is duplicated after a 1 bp gap. The third 13 bp direct repeat ("b") is separated from "a" by a single base pair and is not directly involved in catalysis; it rather serves as a modulator, for instance, by acting as an Flp entry site. While inversion and excision reactions are also feasible with a 34 bp minimal variant site consisting of the inverted repeat and spacer only, a integration reaction benefits from or even requires the presence of the extra Flp-recombinase binding element[44,43,49].

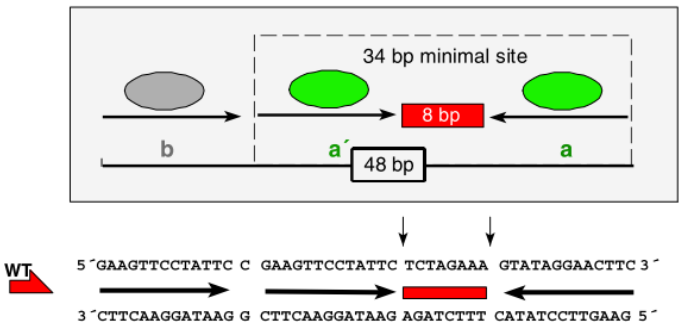


Figure 1.6. Architecture of a wild-type (WT) 48 bp FRT site and its 34 bp minimal variant. FRT site consists of two inverted 13 bp repeats ("a,a") (green ovals) that serve as binding sites for Flp-recombinase arranged about an 8 bp spacer. The asymmetric 8 bp spacer (red box) is the region where breaking and rejoining events occur. The third direct repeat ("b") (gray oval) serves as a Flp entry site. (Adapted from [44]).

2. Aims

The main objective of this work is the production of HCV-like particles and the development of a production platform compatible with industrial production requirements.

This work is, then, divided in two parts. In the first part the evaluation two different cell substrates – HEK293 and HuH-7 - for HCV-LPs production, using cassettes constructed with lentiviral backbone that code HCV genes. HCV-LPs production and purification was assessed.

In the second part, a stable cell line using defined gene expression loci by recombinase-mediated cassette exchange (RMCE) method was developed. This method takes advantage of predictable expression of the gene of interest at a pre-characterized genomic locus. Thus, this strategy may open the possibility to generate an engineered HEK293 cell line producer of HCV-LPs.

3. Material and Methods

3.1. Plasmids used

Primers and templates for all the plasmids constructed in this work are listed in Tables I and II (Attachments).

Lentiviral vectors production

pMD$_2$G codes for the envelope G glycoprotein of the vesicular stomatitis virus (VSV-G) under the control of cytomegalovirus (CMV) promoter.

pMDLg/pRRE is 3rd generation lentiviral packaging plasmid codifying for Gag-Pro-Pol polyprotein and RRE.

pRSV-REV is a 3rd generation lentiviral packaging plasmid that contains Rev protein.

All these plasmids were kindly provided by Dr. Didier Trono (Addgene, USA) and used for transient lentiviral vector production of *pRRLSin* derived vectors, by transfection as reported in *Dull et al.*[42]

Lentiviral transgenes

The plasmids described below were constructed using *pRRLSin* backbone, a self-inactivating (SIN) third-generation lentiviral vector isolated from *pRRLSin GFP-S10*, kindly provided by Miguel Guerreiro (ACT Unit IBET/ ITQB NOVA, Portugal). This backbone contains an ampicillin resistance marker for plasmid amplification in *E.coli*, long-terminal repeats (LTRs), a packaging signal (Ψ) for RNA encapsidation in lentiviral particles, RRE element, and a cPPT for efficient nuclear import/export[38]. After cPPT region each *pRRLSin* plasmid has a different cassette with specific DNA elements to drive the expression of different genes of interest and associated mammalian selection markers. Following the mammalian selection gene all *pRRLSin* vectors have a WPRE regulatory element to increase mRNA stability and a SV40 polyadenylation site to terminate RNA transcription. The different *pRRLSin* plasmids constructed are:

pRRLSin_Core codes for the HCV *Core*, under the regulation of the CMV promoter and an encephalomyocaerditis virus-internal ribosome entry site (EMCV-IRES) drives *neomycin* resistance gene expression.

pRRLSin_E1E2 codes for both HCV envelope glycoproteins, *E1* and *E2*, under the control of the CMV promoter and an EMCV-IRES drives *zeocin* resistance gene expression.

pRRLSin_p7_NS2 codes for HCV non-structural proteins *p7* and *NS2* under the control of hEF1/HTLV chimeric promoter and an EMCV-IRES drives *hygromycin* resistance gene expression.

pRRLSin_NS3_NS4a codes for HCV non-structural proteins *NS3* and *NS4a* under the control of the SV40 promoter and an FMDV-IRES element drives *blasticidin* resistance gene expression.

pRRLSin_NS4b_NS5a codes for HCV non-structural proteins *NS4b* and *NS5a* under the control of hPGK promoter and an EMCV-IRES drives *puromycin* resistance gene expression.

Cassette exchange

pZeoCre contains *Cre* recombinase gene under a CMV promoter.

pTagLoxP_GFPZeo is a Cre-mediated cassette exchange tagging vector constructed by Ana Isabel Almeida (ACT Unit IBET/ ITQB NOVA, Portugal), that contains two loxP sites. This vector drives the expression of a *GFPZeo* gene that was amplified from *pSELECT-GFPZeo-LacZ* (InvivoGen, USA) by PCR and cloned into *pTagLoxP-mcs* designed by Ana Coroadinha.

pTarLoxP_E1E2 is a Cre-mediated cassette exchange targeting vector constructed by Hugo Soares (ACT Unit IBET/ ITQB NOVA, Portugal) from *pTarLoxP* backbone plasmid. This vector contains a CMV promoter, a β-globin that increase mRNA stability, two loxP sites and *E1* and *E2* genes amplified from pEPX145-71 (Epixis, France), a SV40 enhancer conjugated with FermH promoter and mEF1 5'UTR as promoter for puromycin expression.

pSVFlpe contains *Flp* recombinase gene under a SV40 promoter[51].

pTagFRT_mCherry is a Flp-mediated cassette exchange tagging vector contains two FRT sites, a wild-type FRT (Fw) site and a spacer mutant FRT site (F5) with hygromycin resistance gene followed by an ATG defective neomycin phosphotransferase gene (Δ*neo*). The construction contains a *mCherry* gene, amplified from *pRSV-CherryPuro* by PCR and cloned into NotI and PacI excised *pTag*, an in-house constructed plasmid.

pTarFRT_Core is a Flp-mediated cassette exchange targeting vector derived from *pEmMFG* backbone containing a Fw FRT and a F5 FRT, where GFP gene was removed by inverse PCR and replaced by HCV *Core* gene amplified from *pGEM_Core* by PCR.

Retrovirus production

pMD$_2$G as described above (letiviral vectors production).

pMLV-GP codes for murine leukemia virus (MLV) under the control of hEF1/HTLV chimeric promoter.

3.2. Plasmids construction

3.2.1. Cloning procedures

3.2.1.1. Polymerase chain reaction (PCR)

All PCR reactions were performed in a *Biometria® T3Personal Thermocycler* (Biometria, Germany) with a proof-reading *Phusion High-Fidelity* DNA Polymerase (Finnzymes, Finland), using PCR conditions appropriate for each fragment. The oligonucleotides used for PCR were custom-made by Sigma-Aldrich (USA) (Tables I and II).

3.2.1.2. DNA fragment enzymatic restriction

Restriction reactions were incubated 3 hours or overnight at 37°C. The enzymes (New England Biolabs, USA) in Tables I and II (Attachments) were used with the appropriate buffers according to the manufacturer's instructions.

3.2.1.3. DNA fragment purification

All the generated fragments were isolated by agarose gels (NZYTech, Portugal) 0.7% (w/v) prepared in 1x TAE buffer (Tris-Acetate-EDTA) (Qiagen, Germany) and 0.5 µL/mL *RedSafe™ Nucleic Acid Staining Solution* (INtRON Biotechnology, USA) was added before pouring the gel. Subsequently, DNA fragments were visualized using *GelDoc™ XR+* system (BioRad, USA) and purified with *NucleoSpin® Gel and PCR Clean-up* kit (Macherey-Nagel, Germany), according to the manufacturer's instructions.

3.2.1.4. Vector and insert ligation

Vector-insert ligations were performed using *In-Fusion® HD Cloning Kit* (Clontech Laboratories, USA) following manufacturer's instructions.

3.2.2. Bacterial strains

Escherichia coli (*E.coli*) Stellar™ (Clontech, USA) and One Shot® Stbl3™ (Invitrogen, USA) competent cells were used for the production of the DNA plasmids. Transformation procedures were carried under manufacturer's instructions. The agar and liquid cultures were performed with *Luria Broth* media (LB) (Fast-Media® LB from Invivogen, USA) and *Terrific Broth* media (TB) (Fast-Media® TB from Invivogen, USA), respectively, supplemented with the appropriated antibiotic. The media was prepared using milliQ water (Milli-Q® from Merck Millipore, USA), according to manufacturer's instructions.

3.2.3. Plasmid purification and quality control

Final plasmid production and purification was performed in different scales. Small-scale, medium-scale and large-scale production and purification were performed using *GeneJET Plasmid*

Miniprep Kit (Thermo Scientific, USA), *ZymoPURE™ Plasmid Midiprep* (Zymo Research, USA) and *Genopure Plasmid Maxi Kit* (Roche Applied Science, Germany), respectively, following manufacturer's instructions. Working DNA stock solution for each plasmid were generated and stored at -20°C. The DNA concentration was determined in *Nonodrop 2000C spectrophotometer* (Thermo Scientific, USA). Plasmid purity was determined by the Abs_{260nm}/Abs_{280nm} and Abs_{260nm}/Abs_{230nm} ratios and plasmid integrity with enzymatic restriction analysis assessed in 0.7% (w/v) agarose gels (NZYTech, Portugal) prepared in 1x TAE buffer (Qiagen, Germany). All plasmids were sequenced using GATC Biotech services (Constance, Germany).

3.3. Cell lines and culture conditions

HEK293 is a Human Embryonic Kidney cell line (ATCC CRL-1573). HEK293T (ATCC CRL-11268) is a HEK293 derived cell line expressing large T antigen from SV40. HuH-7 (JCRB0403) is a Human Hepatoma cell line that was originally take from a liver tumor. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, UK) supplement with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco, UK) and maintained in an incubator at 37°C in a humidified atmosphere containing 8% CO_2.

3.4. Cell number and viability

Cell concentration and viability were determined by the trypan blue exclusion method using a 0.1% (v/v) solution prepared in Phosphate Buffer Saline (PBS) (Gilbo, UK) and a Fuchs-Rosenthal hemocytometer (Brand, Germany) observed on an inverted microscope (Olympus, Japan). Cells with damaged membranes stained blue and viable cells remain colorless.

3.5. Lentiviral vector production

For transient lentiviral vector production, the 3rd generation lentiviral packaging system was used[42]. HEK293T cells were seeded at 5 x 10^5 cells/cm^2 in 75cm^2 t-flask. Polyethylenimine (PEI, linear 25 kDa) (Polysciences, Germany) transfection was carried out 24 hours later with a mixture of *pMD$_2$G* (for the envelope), *pRSV-REV* and *pMDLg/pRRE* (providing the packaging functions), and *pRRLSin* derived vectors (section 2.1) providing the gene of interest (transgene) at 1:1.5 ratio of DNA:PEI. The DNA ratio used was 1:0.5:1:2, respectively. 24 hours post-transfection, culture medium was replaced with the original volume (15 mL). Culture medium containing the lentiviral vectors was collected after an additional 24 hours production period. All viral vectors supernatant was filtered through 0.22 µm pore-sized cellulose acetate filter, aliquoted and stored at -80°C until further use.

3.6. Lentiviral transduction

HCV genes were delivered by lentiviral vector infection. HEK293 and HuH-7 cells were seeded at a density 5 x 10^5 cells/well 24 hours before infection in 6-well plates. Transduction was performed by removing the cell supernatant and infecting with 600 µL of viral suspension prepared as described

in section 3.5 in the presence of 8 µg/mL of polybrene (Sigma-Aldrich, USA). Cells were incubated at 37°C for 24 hours. 24 hours post-infection, the culture supernatant was exchanged for fresh culture DMEM. Two days after infection, cells were amplified to 75 cm^2 t-flasks in DMEM supplemented with the respective selection antibiotic (according to each plasmid). The cells were maintained in culture for 21 additional days with culture medium exchange every 3-4 days.

3.7. RNA extraction and gene expression quantification by Real-Time quantitative PCR

To analyze HCV components expression total RNA was extracted using *QIAamp® RNeasy Mini Kit* (Qiagen, USA) according to the manufacturer's instructions. Ribosomal protein L22 (RPL-22) was chosen as a control housekeeping gene. Primer sequences for control and HCV genes are given in Table III (Attachments). The reverse transcription of total RNA was performed according to *Transcriptor High Fidelity cDNA Synthesis Kit* (Roche Applied Science, Germany) protocol for cDNA synthesis using 2 µg of total RNA and oligo dT primer for total mRNA reverse transcription. The reverse transcribed product was aliquoted and stored at -20ºC until further processing. SYBR Green I dye chemistry was used to detect the PCR products using *LightCycler® 480 SYBR Green I Master* (Roche Applied Science, Germany) according to the manufacturer's instructions using *LightCycler® 480 Real Time PCR System* (Roche Applied Science, Germany). Two independent biological replicates were analyzed using $2^{-\Delta Ct}$ method for relative gene expression analysis[53].

3.8. HCV-LPs production and purification

For HCV-LP production and purification, cells were seeded in 225 cm^2 t-flasks and cultured until 80% of cellular confluence was reached. Cells were harvested and centrifuged at 300 g for 10 minutes at 4°C. Cells were resuspended in 20 mL of a hypotonic solution supplemented with 1x protease inhibitor cocktail (Roche Applied Science, Germany) and incubated on ice for 30 min. Cells lysis was performed with 5 freeze/thaw cycles with liquid nitrogen. Cell debris were removed by centrifugation (14000 g for 10 min at 4°C). Cleared supernatant was collected and completed with 30 mL PBS. Supernatant was then filter through 0.20 µm for further clarification and centrifuged in a *45 Ti rotor* (Beckman Coulter, USA) at 40000 *rpm* (≈185000 *g*) for 2 h at 4°C. The pellet was resuspended in 1.5 mL PBS. Pellet resuspension was added on top of a 30% (v/v) sucrose cushion[54] and centrifuged in a *90 Ti rotor* (Beckman Coulter, USA) at 60000 *rpm* (≈300000 *g*) for 2 h at 4°C. Virus pellet was resuspended in 1x Tris-buffered saline (TBS) solution.

3.9. Western blot

For protein electrophoresis, *NuPAGE® electrophoresis system* (Invitrogen, USA) was used. Samples were prepared in denaturing conditions according to manufacturer's instructions and heated at 70°C for 10 minutes. Samples were resolved on a *NuPAGE®* 4-12% Bis-Tris gel with

NuPAGE® MES SDS Running Buffer, at 180V for 40 minutes. Protein transfer into PVDF membrane was performed in *Trans-Bot® Turbo™ Transfer System* (Bio-Rad, California, USA) for 30 minutes, according to manufacturer's instructions. Membranes were blocked with 5% (w/v) dry milk in 1x PBS, for at least 1 hour at room temperature and incubated overnight with primary antibody diluted in 1% BSA in PBS at room temperature with gentle agitation. The primary antibodies used were mouse monoclonal anti-HCV Core (C7-50) (Santa Cruz, USA) at 1:50 in PBS, mouse monoclonal anti-HCV E1 (BDI198) (Acris Antibodies GmbH, Germany) at 1:50 in PBS and mouse monoclonal anti-HCV E2 (4F6/2) (Austral Biologicals, USA) at 1:500 in PBS. Membranes were washed with 1x PBS for 10 minutes (3 times) and incubated with secondary antibody, anti-mouse IgG HRP conjugate (NA931) (GE Healthcare, UK) at 1:1000 in PBS, for 2 hours at room temperature. Chemiluminescence detection was performed with *Amersham™ ECL™* prime western blotting detection reagent (GE Healthcare, UK), according to manufacturer's instructions, and analyzed under *ChemiDoc™ XRS system* (Bio-Rad, USA).

3.10. Cell transfection with tagging cassette and drug selection

HEK293 clones #1, #2 and #3 previously tagged with the *pTagLoxP_GFPZeo* cassette were kindly provided by Ana Isabel Almeida (ACT Unit IBET/ ITQB NOVA, Portugal). Cells were seeded in 6 well plates at $5X10^5$ cells/well. After 24 hours, transfection was performed using PEI (Polysciences, Germany) to insert *pTagFRT_mCherry* cassette at 1:1.5 ratio of DNA:PEI. Cells were incubated at 37ºC overnight after which medium was exchanged. 48 hours post-transfection, cells were analyzed for mCherry fluorescence by flow cytometry and were cultured for 21 additional days in hygromycin (300 µg/mL) (InvivoGen, USA) containing medium, exchanging culture medium at each 3-4 days. Hygromycin resistante cell populations were cloned by limiting dilution to isolate single cells which were cultured and expanded. Different cell clones with homogeneous mCherry expression and cassette integration pattern were screened by flow cytometry and RT-qPCR.

3.11. Fluorescence signal detection

For GFP and mCherry fluorescence signal detection, HEK293 cells tagged with a *pTagLoxP_GFPZeo* cassette and a *pTagFRT_mCherry* cassette were trypsinised and resuspended in PBS at a density of $2x10^6$ cells/mL. Cell counts were performed on *CyFlow®space* (Sysmex Partec, Germany). The excitation wavelengths for GFP and mCherry were 488 nm and 587 nm and emission were detected at 507 nm and 610 nm[55], respectively. The cells were considered fluorescent when the measured fluorescence intensity exceeded the signal obtained with non-transfected cells.

3.12. Tagging copies quantification by Real-Time quantitative PCR

For the quantification of integrated tagging copies into the cell genome, genomic DNA was isolated from $5x10^6$ cells using the *QIAamp® DNA Mini and Blood Mini Kit* (Qiagen, USA) according to manufacturer's instructions. To estimate the number of copies *per* cell, genomic DNA was quantified using *Nanodrop 2000C spectrophotometer* (Thermo Scientific, USA) and 200 ng of DNA was used as template for RT-qPCR. Specific primers for hygromycin gene (Table III) were used on *LightCycler® 480 Real-Time PCR System* (Roche Applied Science, Germany) according to *LightCycler 480 SYBR Green I Master* (Roche Applied Science, Germany) PCR kit. All data was analyzed using $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis[53]. After normalization to the housekeeping gene *RPL22*, the number of copies *per* cell was quantified using a standard curve and compared to a cell line with a single copy integrated genome. Single copy was considered for values between 0.6 and 1.4[56].

3.13. Retrovirus production

To determine the packaging signal efficiency of the *pTagFRT_mCherry* cassette incorporated into mRNA of the cells, clones were seeded in 6 well plates at $5X10^5$ cells/well. 24 hours later, transient transfection was performed using PEI (Polysciences, Germany) at 1:1.5 ratio of DNA:PEI and with 2 µg of *pMD₂G* and 2 µg of *pMLV-GP*. After 24 hours, culture medium containing the retrovirus was collected. HEK293T cells were transduced and analyzed for mCherry fluorescence intensity.

3.14. Cassette exchange

For Cre-mediated cassette exchange, clones #206 and #311 were seeded in 6 well plates at $5X10^5$ cells/well. 24 hours later, transfection was performed using PEI (Polysciences, Germany) at 1:1.5 (w/w) ratio of DNA:PEI or calcium phosphate ($CaPO_4$) with 1 µg of targeting plasmid (*pTarLoxP_E1E2*) and 3 µg of *pZeoCre*. 48 hours post-transfection, cells were analyzed for cassette exchange by flow cytometry and were selected with puromycin (1 µg/mL) (InvivoGen, USA). Cells were cultured for 21 days and medium exchanged at each 3-4 days. Puromycin resistance cell populations were cloned by limiting dilution to isolate single cells which were cultured and expanded. One cell clone (#311_F8) with no green expression was expanded and frozen.

For Flp-mediated cassette exchange, #311 parental and clone #311_F8 were seeded in 6 well plates at $5X10^5$ cells/well. 24 hours later, transfection was performed equally as described above with 4 µg of targeting plasmid (*pTagFRT_mCherry*) and 12 µg of *pSVFlpe*. 48 hours post-transfection, cells were analyzed for cassette exchange by flow cytometry and were selected with neomycin (500 µg/mL) (InvivoGen, USA). Cell were cultured for 21 days and medium exchanged at each 3-4 days. Neomycin resistance cell populations will be cloned by limiting dilution to isolate single cells, cultured and expanded. One cell clone with no red expression will be expanded and frozen.

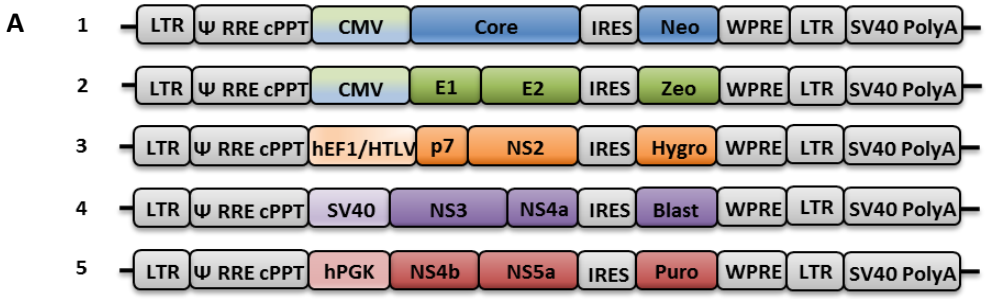
4. Results

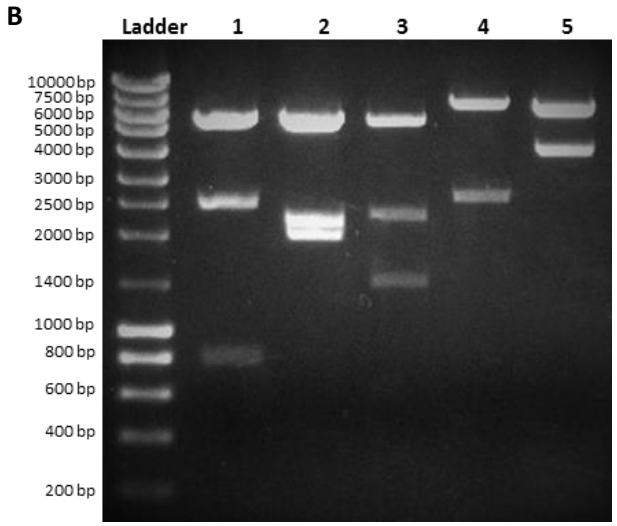
4.1. Evaluation of different cell substrates for HCV-LP production

The production of a HCV-LP vaccine is dependent on the development of a HCV-LP producer cell line[14]. It is widely known that several factors may affect quality and production yields of viral particles. Therefore, this work begins by testing two different cell-lines (HEK293 and HuH-7) to produce HCV-LP.

4.1.1. Vectors construction and cells transduction

To develop a cell line for stable production of HCV-LP composed of HCV proteins, lentiviral vectors containing HCV genes - *Core*, *E1* and *E2*, *p7* and *NS2*, *NS3* and *NS4a*, *NS4b* and *NS5a* - were constructed (Figure 4.1). Lentiviral vectors were produced by transient transfection of HEK293T cells.





Plasmids	EcoRI and XhoI restriction patterns
1	5497 bp + 2441 bp + 774 bp
2	5497 bp + 2147 bp + 1952 bp
3	5497 bp + 2235 bp + 1381 bp
4	6947 bp + 2559 bp
5	6083 bp + 3850 bp

Figure 4.1. HCV lentiviral vectors. A. Schematic presentation of the HCV lentiviral vectors constructed. LTR, long-terminal repeat; Ψ, packaging signal; RRE, rev responsive element; cPPT, polypurine sequence; IRES, internal ribosome entry site; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; and SV40 polyadenylation site. CMV, hEF1/HTLV, SV40 and hPGK are promoters. Neomycin (Neo), zeocin (Zeo), hygromycin (Hygro), blasticidin (Blast) and puromycin (Puro) are selection antibiotics for mammalian (not in scale). **B. Vectors restriction analysis.** Electrophoresis showing a restriction-specific pattern of the plasmids, when using the *EcoRI* and *XhoI* restriction enzymes. 1-*pRRLSin_Core*; 2-*pRRLSin_E1E2*; 3-*pRRLSin_p7_NS2;* 4-*pRRLSin_NS3_NS4a;* 5-*pRRLSin_NS4b_NS5a*. Ladder-NZYDNA Ladder III (NZYTech, Portugal); (bp, base pairs).

HEK293 and HuH-7 cells were sequentially transduced with lentiviral vectors transducing HCV genes (*Core*, *E1E2* and *p7NS2*) and selected with the corresponding antibiotic resistance gene.

4.1.2. Expression of HCV genes and proteins

HCV *Core*, *E1* and *E2* genes provide all structural components needed for HCV-LP formation. Additionally, evidences suggest that *p7* and *NS2* are essential for viral particles assembly and function[17,27]. Before assessing the production of HCV-LP, HCV genes and proteins expression in HEK293 and HuH-7 transduced cell populations was determined by RT-qPCR and Western blot, respectively.

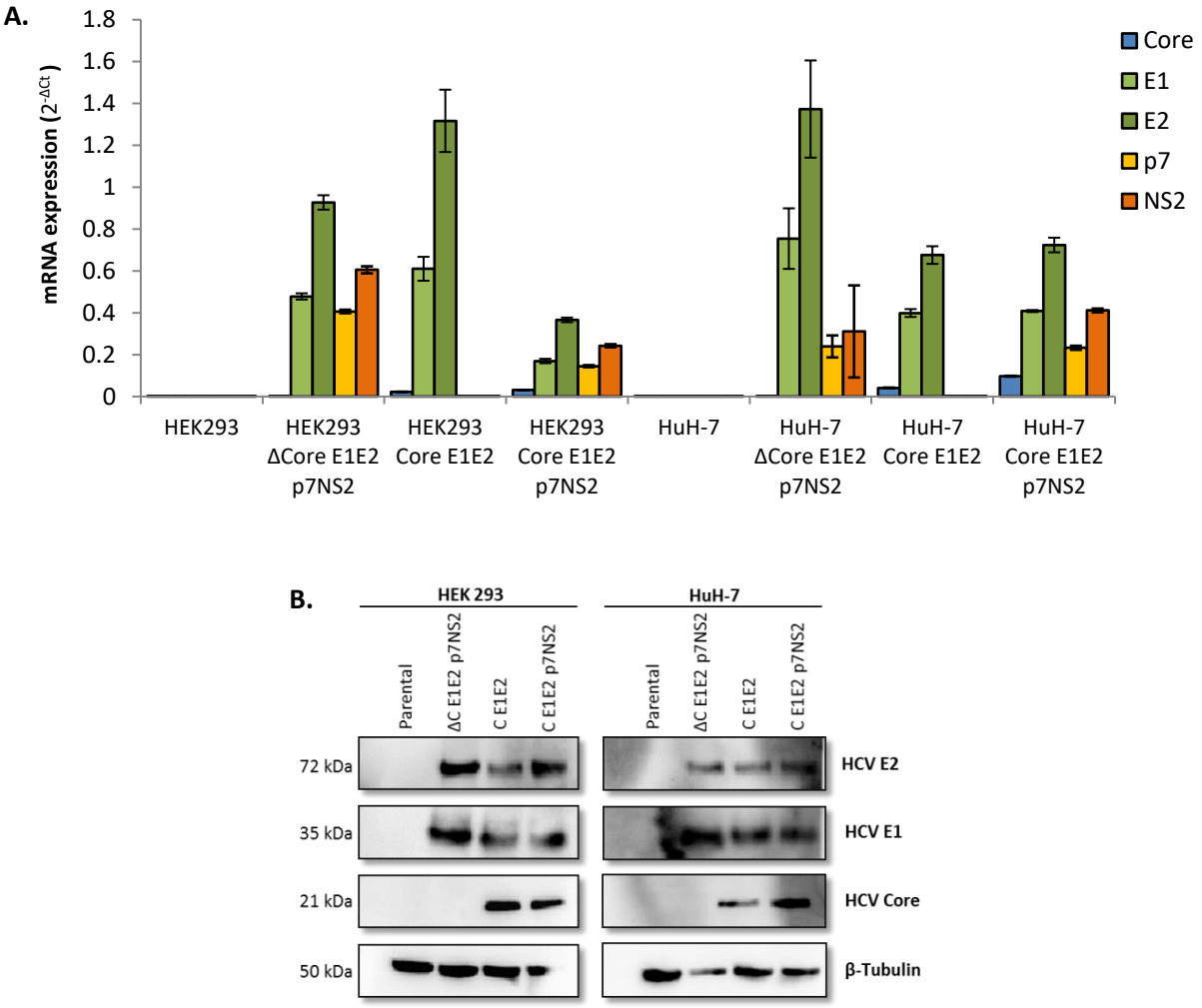


Figure 4.2. Expression of HCV genes and proteins in lentivirus transduced cell lines. Cell lines used for both analyses were HEK293 and HuH-7 as negative control (without HCV genes); HEK293 and HuH-7 ΔC E1E2 p7NS2 as Core negative control (without Core); HEK293 and HuH-7 Core E1E2 as p7NS2 negative control (without p7NS2) and HEK293 and HuH-7 Core E1E2 p7NS2. **A. Analysis of the HCV genes expression by RT-qPCR.** Numbers on the x-axis correspond to cell lines. Numbers on the y-axis indicate the mRNA expression compared to HEK293 and HuH-7 given by mRNA quantification by RT-qPCR using primers for *Core, E1, E2, p7* and *NS2* genes. The genes expression was quantified after normalization to a control gene (*RPL22*) using the $2^{-\Delta Ct}$. Genes expression levels are shown as average ± standard deviation of four technical replicates and two biological replicates. **B. Analysis by Western blot of HCV proteins (Core, E1 and E2) expression**. Protein extracts obtained from LV transduced cells. Core (21 kDa), E1 (35 kDa) and E2 (72 kDa) expression detected by an anti-Core antibody, an anti-E1 antibody and anti-E2 antibody, respectively. The β-tubulin (50 kDa) content was also analyzed to verify that equal levels of protein extracts have been loaded and it expression detected by an anti-β Tubulin antibody.

As shown in Figure 4.2A, parental HEK293 and HuH-7 cell lines do not express any of the viral genes while mRNA for all viral components was detected in all transduced cells. It is possible to observe a great variation in gene expression among tested cells, this variation might be explained by the analysis of heterogeneous cell populations rather than homogeneous populations derived from a single-cell clones. Of note is the relative low expression of HCV *Core* gene in both HEK293 and HuH-7 cell populations. This observation, even though relevant, does not prevent the expression of HCV Core protein in detectable amounts (Figure 4.2B). In parallel, protein expression of viral components was assessed by Western Blot analysis. As visible in Figure 4.2B all viral components (Core, E1 and E2) are expressed at similar levels in all transduced cell populations. HCV p7 and NS2 protein expression could not be assessed since no commercial antibodies are available for the studied HCV strain (H77).

4.1.3. HCV-LP production and purification

After validating the expression of the viral components in transduced HEK293 and HuH-7 cells, we evaluated the production of fully assembled HCV-LP. All cell lines and controls were seeded in 225 cm^2 t-flasks and cultured until 80% of cellular confluence was reached. Culture medium was then replaced with fresh one. HCV-LPs produced during the subsequent 24 hours were harvested and purified by sucrose cushion centrifugation and analyzed for Core, E1 and E2 presence by Western blot.

As visible in Figure 4.3 higher amounts of HCV envelope antigens are detected in samples where Core protein is present in higher amounts for both cell lines.

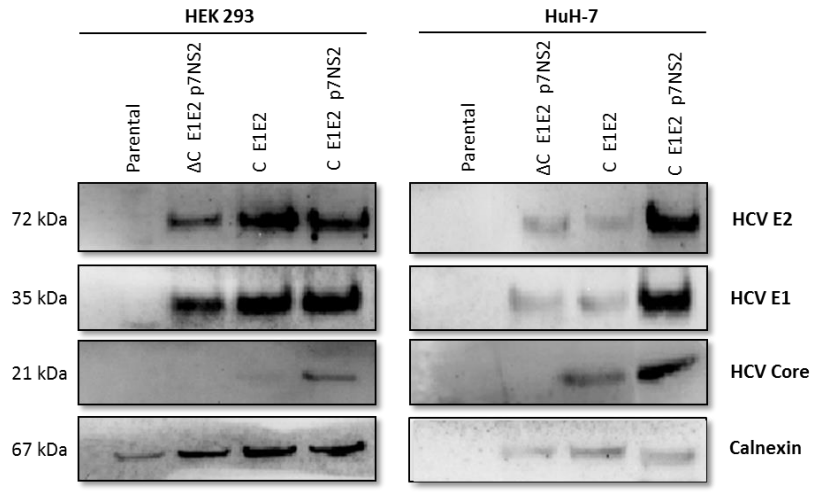


Figure 4.3. Characterization of HCV-LP produced. Western blot profile of HCV-LP by Core, E1 and E2 expression. Cell extracts obtained from LV transduced cells after sucrose cushion centrifugation. Core (21 kDa), E1 (35 kDa) and E2 (72 kDa) expression detected by an anti-Core antibody, an anti-E1 antibody and anti-E2 antibody, respectively. The Calnexin (67 kDa) content was also analyzed to verify that equal levels of protein extracts have been loaded (anti-Calnexin antibody).

4.1.4. Influence of serum on HCV-LP production

Recent studies have shown the negative impact of FBS for HCV infectivity[57,58,59]. Therefore, the effect of FBS on HCV-LP production was determined by analyzing the impact of decreasing serum concentration in the culture medium. The serum concentration in the culture medium was reduced from 10% (v/v) to 5% (v/v) and to 2% (v/v) for HEK293 and HuH-7 CoreE1E2p7NS2 cell lines.

In collaboration with Downstream Process Development Laboratory (ACT Unit IBET/ ITQB NOVA, Portugal), the presence of HCV-LP in culture supernatants was determined by size-exclusion chromatography onto a column Superdex S-200, as described elsewhere[60].

The data indicate that HCV-LP production decreased when serum concentration is reduced for both producer lines cells (Figure 4.4). This was most evident for 2% (v/v) FBS concentration.

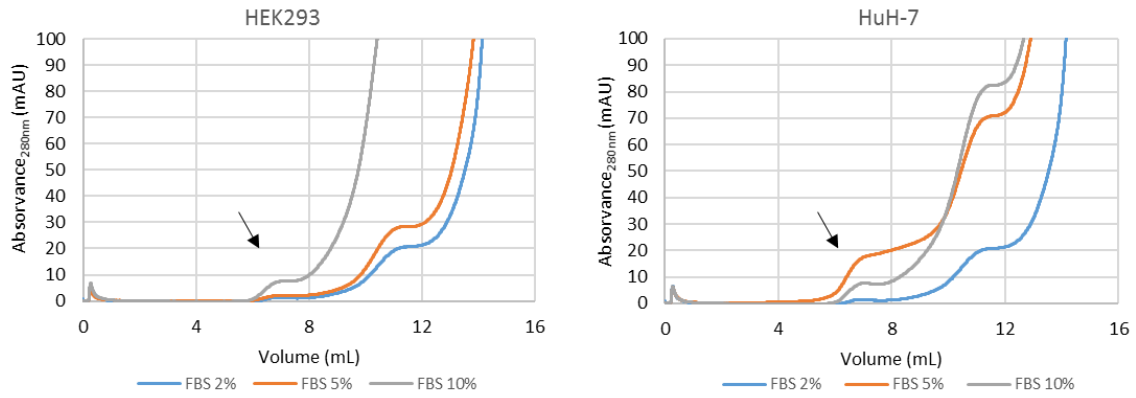


Figure 4.4. Effect of serum on HCV-LP production. Separation of HCV-LP produced by HEK293 (A) and H HuH-7 (B) cells transduced with *Core, E1, E2, p7* and *NS2* transgenes by size-exclusion chromatography. Culture medium of both cell lines was treated with 10% (v/v) FBS (gray line), 5% (v/v) FBS (orange line) and 2% (v/v) FBS (blue line). The $A_{280\ nm}$ of each fraction was determined by UV.

4.2. Development of a stable cell line using defined gene expression loci

A suitable cell line for large-production of biopharmaceuticals must fulfill a series of legal and industrial requisites. Therefore, a defined and controlled introduction of viral components is desirable over random integration.

Site-specific recombination is an excellent candidate methodology to overcome some of the issues associated with traditional plasmid transfection and lentiviral transduction, namely loss of gene expression over time, integration of multiple copies of the same gene, integration in low expression locus or the disruption of essential genes[51,61].

This work starts with the generation of a cell line suitable for site-specific recombination using both Cre-recombinase and Flipase-recombinase enzymes. The overall strategy is described in Figure 4.5.

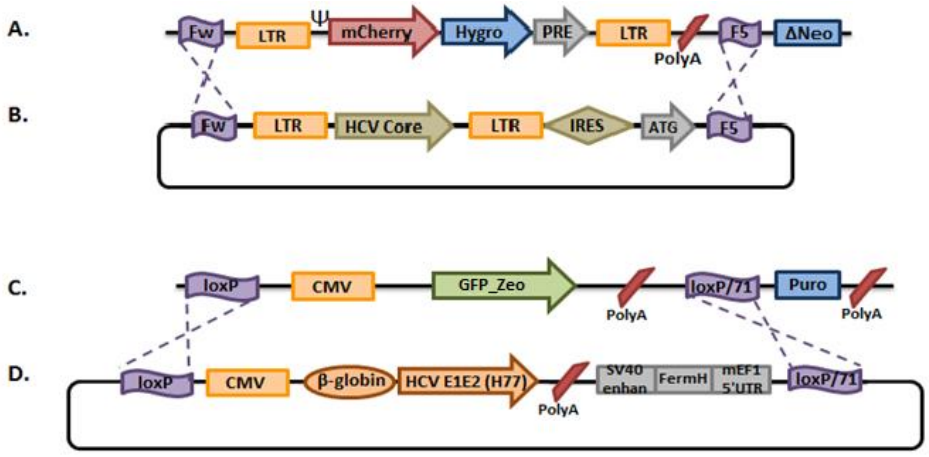


Figure 4.5. Strategies for the cassette exchange by Flp- and Cre-mediated recombination. A. Tagging plasmid (*pTagFRT_mCherry)* containing a *mCherry* reporter gene, *hygromycin* resistance gene and two FRT sites, a wild-type (Fw) and a mutant (F5), followed by a defective *neomycin* gene. **B.** Targeting plasmid (*pTarFRT_Core*) containing the two FRT sequences flanking the HCV *Core* gene and an ATG sequence that will restore the *neomycin* gene in the tagged clone after Flp-mediated exchange. **C.** Tagging plasmid (*pTagLoxP_GFPZeo*) containing a fusion gene that encodes *GFP* and resistance to *zeocin* and two loxP sites, a wild-type and a mutant (loxP/71). **D**. Targeting plasmid (*pTarLoxP_E1E2*) containing the two loxP sequences flanking the HCV *E1E2* (H77) gene and an internal promoter that will drive the expression of the promoterless *puromycin* gene after after Cre-mediated exchange.

4.2.1. Construction and validation of tagging cassette

The constructed *pTagFRT_mCherry* tagging cassette contains a *mCherry* gene and two FRT sites to allow cassette exchange by Flipase enzyme. *mCherry* was chosen as reporter gene for the tagging cassette because its excitation and emission spectra do not overlap with those of GFP[55] which is present in the *pTagLoxP_GFPZeo* tagging cassette previously introduced in these cells.

To quickly evaluate the functionality of *pTagFRT_mCherry* cassette, HEK293T cells were transiently transfected with the expression cassette and mCherry fluorescence was determined by flow cytometry and fluorescence microscopy (data not shown). The transfected HEK293T cells showed high mCherry fluorescence when compared with non-transfected HEK293T cells, revealing *mCherry* gene expression and consequent functionality of both promoter and reporter gene regions.

4.2.2. Transfection and efficiency of tagging cassette insertion

Before starting cell line development efforts, we evaluated the sensitivity to hygromycin of HEK293 clones #1, #2 and #3 previously tagged with *pTagLoxP_GFPZeo* cassette (data not shown). This step ensured that no intrinsic hygromycin resistance was present in any of the candidate cell lines therefore validating their use in subsequent experiments.

We then proceed with tagging of *pTagFRT_mCherry* cassette by transfection of all three clones, as described in section 3.10, and screening for *hygromycin* resistance and *mCherry* expression. mCherry expression in transfected cells was visually inspected by fluorescence microscopy 72 hours following the transfection. These, together with non-transfected control, were subjected to hygromycin selection as described. After two weeks in selection, mCherry expression

was determined in surviving cells by visual inspection revealing different mCherry intensity among different cell populations (Figure 4.6). Since PEI has ability to act as a proton sponge that buffers the low pH and potentially induces the rupture of the membrane, releasing PEI/DNA complex into the cytoplasm, an efficient transfection was expected[62].

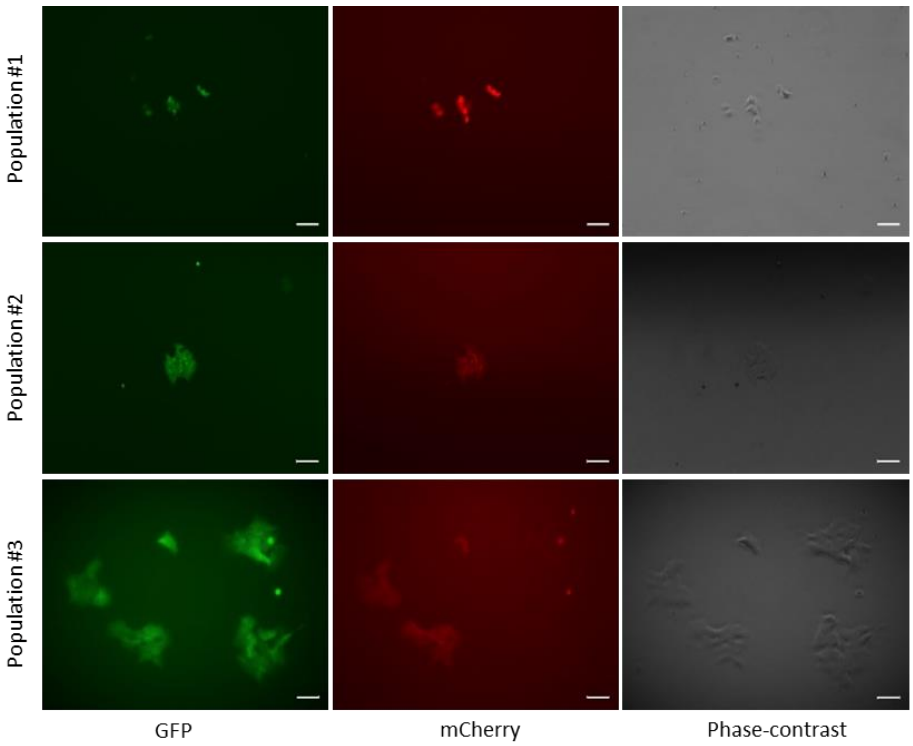


Figure 4.6. Efficiency of tagging cassette insertion. Fluorescence microscopy analysis showing cells previously tagged with the *pTagLoxP_GFPZeo* cassette transfected with *pTagFRT_mCherry* cassette expressing *GFP* and *mCherry* reporter genes, respectively. Scale bars: 100µm.

4.2.3. Limiting dilution and clone screening

Aiming to develop homogeneous cell clones expressing similar levels of *mCherry*, a limiting dilution was performed to isolate single cells which after amplification culture originate a homogeneous cell population. For the clones to grow from a single cell to a confluent well from a 96 well plate a whole month was needed; in the end we obtained 16 clones. At confluence, clones were successively cultured to higher surface growing areas until reaching a confluent 150 cm^2 t-flask. At this stage, individual clones were cryopreserved.

Selected hygromycin resistant clones were analyzed by flow cytometry to determine percentage of mCherry positive cells and compared with non-transfected cells. As visible in Figure 4.7, 10 clones (#102, #103, #206, #301, #302, #304, #305, #306, #310 and #311) show high percentage of mCherry positive cells.

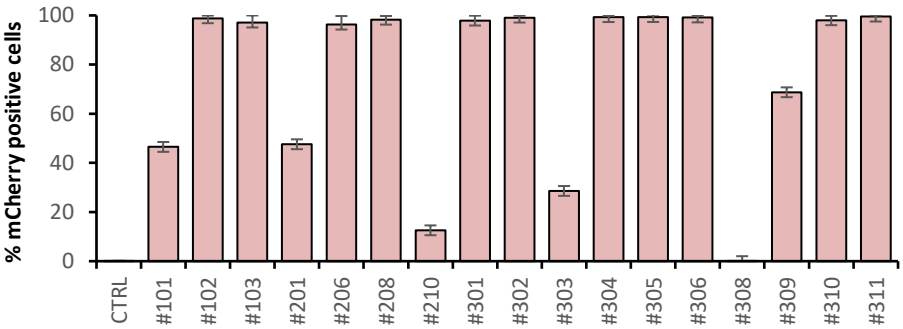


Figure 4.7. Percentage of mCherry positive cells. Flow cytometry analysis showing the percentage of mCherry positive cells of 16 clones with *pTagFRT_mCherry* cassette. Non-transfected HEK293 cells (CTRL) as mCherry negative control. The percentage of mCherry positive cells is represented by red bars. Error bars correspond to 2% error, the error associated to the flow cytometry method.

Afterwards, these 10 clones were screened by flow cytometry for fluorescence intensity of both GFP from *pTagLoxP_GFPZeo* cassette and mCherry from *pTagFRT_mCherry* cassette. This screening reveal 1 clone with high mCherry fluorescence (#102), 1 low (#103) and 8 with medium (#206, #301, #302, #304, #305, #306, #310 and #311) (Figure 4.8). As fluorescence intensity can be a combination of gene expression and copy number, we continue cell characterization by determining of the number of *pTagFRT_mCherry* copies which integrate cells genome.

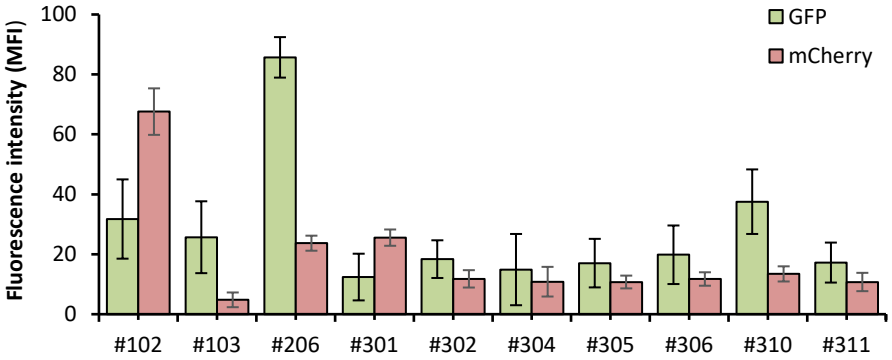


Figure 4.8. Fluorescence intensity of GFP and mCherry-expressing clones. Numbers on the x-axis correspond to tagged clones and numbers on the y-axis indicates GFP and mCherry fluorescence intensity. GPF and mCherry fluorescence is represented by green and red bars, respectively. Fluorescence intensity of GFP and mCherry is shown as average ± standard deviation of three technical replicates.

4.2.4. RT-qPCR analysis of tagged clones

To determine the copy number of tagging construct in the genome of the different clones, RT-qPCR analysis was performed, using specific primers for *hygromycin* gene. *RPL22* was used as housekeeping gene for normalization to DNA content. Four out of the 10 clones showed more than one copy (#102, #304, #305 and #310) and were thus discarded. The remaining clones (#103, #206, #301, #302, #306 and #311) have shown a single copy integration, considering values below 1.4[56] (Figure 4.9).

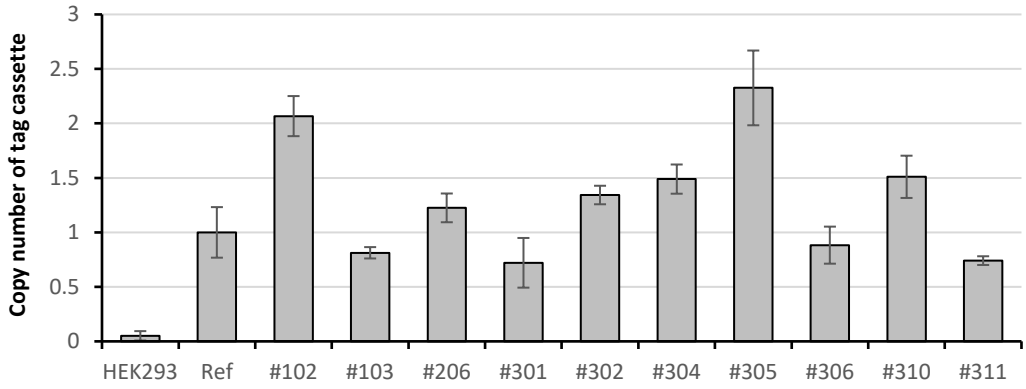


Figure 4.9. Tagging cassette copy number in the genome of the clones. Numbers on the x-axis correspond to tagged clones. Numbers on the y-axis indicate the ratio of copy number relatively to a single copy control (Ref) of targeted transgene given by genomic DNA quantification by RT-qPCR using primers for *hygromycin* gene. The number of copies *per* cell was quantified after normalization to a control gene (*RPL22*) using $2^{-\Delta\Delta Ct}$ method, as defined in section 2.14. The copy number of tagging cassette is shown as average ± standard deviation of three technical replicates. Values below 1.4 were considered a single copy integration. HEK293 as negative control (without *pTagFRT_mCherry* cassette); Ref as positive control (with a single copy).

4.2.5. Packaging signal efficiency

We isolated individual clones with high mCherry expression driven by the integration of single copy of *pTagFRT_mCherry* cassette. We continued with characterization of integrated cassette by evaluating another functional element of the cassette – the packaging signal (Ψ). This sequence allow mRNA coding for mCherry to be incorporated in gamma-retrovirus particles and delivered afterwards to target cells[63].

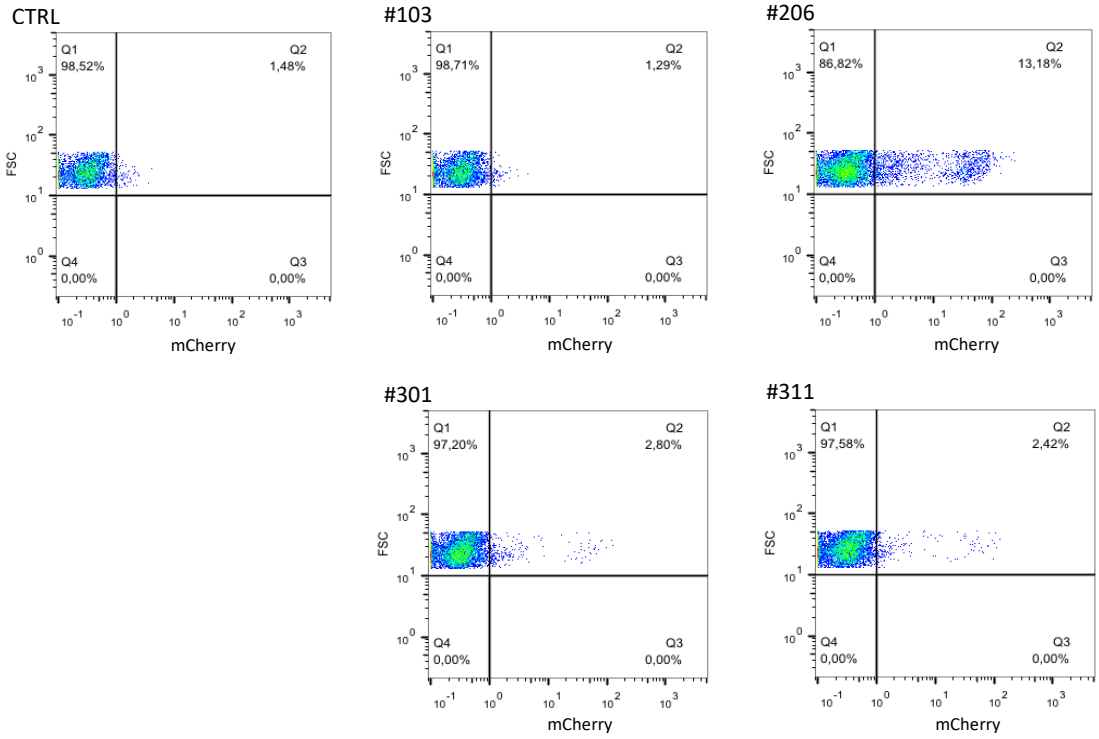


Figure 4.10. Functionality of packaging signal (Ψ). Representative dot plot flow cytometry analysis represents percentage of mCherry^{+} cells of clones #103, #206, #301 and #311 transiently transfected with *pMLV-GP*. Non-transfected HEK293 cells were used as negative control. Q1 - % mCherry negative cells; Q2 - % mCherry positive cells; Q3 and Q4 - cell debris. The values in each selected region are the percentages of gated cells analyzed.

Therefore, 4 clones (#103, #206, #301 and #311) were transiently transfected with a plasmid coding for MLV structural proteins and a second plasmid coding VSV G envelope protein, as described in section 3.13. The capacity to incorporate and deliver mCherry mRNA in target cells was evaluated by flow cytometry. Considering that high mCherry signal correlate with efficient packaging of viral mRNA (Figure 4.10), clones #206 and #311 were selected to proceed for cassette exchange.

4.2.6. Cre-mediated cassette exchange

The clones #206 and #311 were co-transfected with the Cre expressing plasmid and the exchange vector *pTarLoxP_E1E2*, that is flanked by two loxP sites in the same orientation as the genomic tagging. The transfection was performed, comparing two different methods: PEI and calcium phosphate ($CaPO_4$), as described in section 3.14. Untransfected #206 and #311 clones were used as a control of cassette exchange. Puromycin selection was performed and puromycin resistant cells, indicative of Cre-mediated cassette exchange, were only obtained from clone #311.

After expanding resistant cell population, we analyzed cassette exchange efficiency by flow cytometry. This was determined of 7% for both transfection methods, as shown in Figure 4.11.

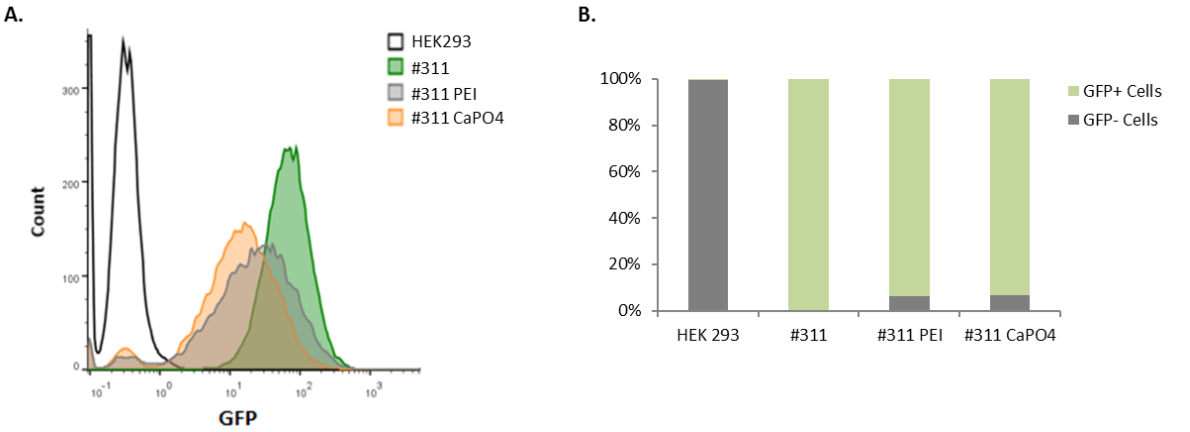


Figure 4.11. Efficiency of Cre-mediated cassette exchange. A. and B. Histograms obtained by flow cytometry analysis showing an efficiency of cassette exchange of clone #311 transfected with *pTarLoxP_E1E2* by PEI (#311 PEI) and calcium phosphate (#311 $CaPO_4$) methods. HEK293 as negative control (without *pTagLoxP_GFPZeo* cassette) and Clone #311 as positive control (with *pTagLoxP_GFPZeo* cassette). GFP^+ cells express GFP and GFP^- cells no express GFP.

We then proceed with limiting dilution to isolate targeted clones, which could be identified by visually screening 96-well plates for cell colonies with positive mCherry signal and negative GFP. We have obtained 1 clone (#311_F8) *per* two 96 well plates (Figure 4.12). After extensive culturing this clone was amplified and cryopreserved.

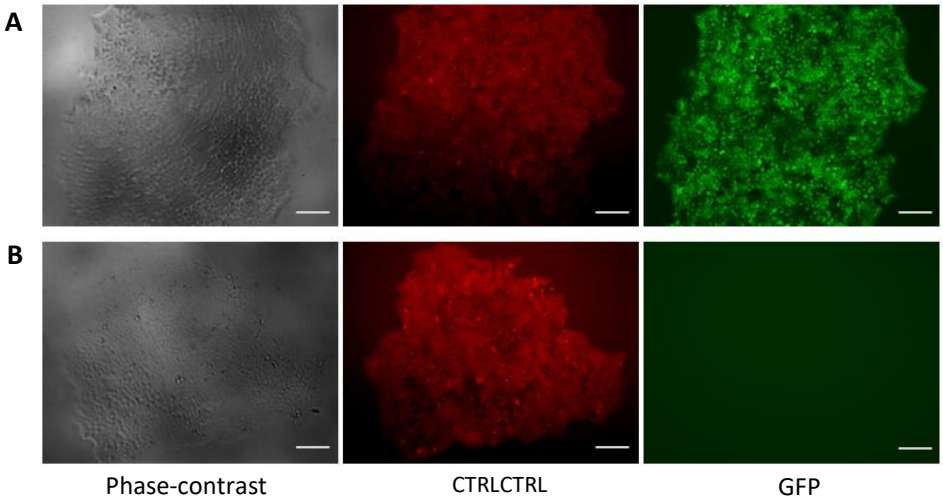


Figure 4.12. Efficiency of Cre-mediated cassette exchange of clone #311_F8. Fluorescence microscopy analysis showing the efficiency of cassette exchange of clone #311_F8 targeted with *pTarLoxP_E1E2* cassette, after 21 days puromycin selection. **A.** #311 cells without cassette exchange. **B.** Clone #311_F8 with cassette exchange, post limiting dilution. Scale bars: 100µm.

4.2.7. Flp-mediated cassette exchange

Thus, parental clone #311 and clone #311_F8 were co-transfected with the Flipase expressing plasmid and the exchange vector *pTarFRT_Core*, using PEI or calcium phosphate ($CaPO_4$), as described in section 3.14. This target cassette codes the ATG starting codon which is lacking in the ATG defective *neomycin* resistance gene in the tagging cassette (*pTagFRT_mCherry*). Unstransfected parental #311 and clone #311_F8 were used as control of cassette exchange. Neomycin selection and clone expansion of resistant cells is still ongoing.

5. Discussion

Hepatitis C Virus (HCV) infects approximately 3% of the world population, being one of the major causes of liver cancer[6]. The increasing demand for a universal, highly effective therapy of chronic HCV infection has placed pressure to develop safe and effective vaccines against HCV to control the global epidemic[14,15]. However, several obstacles hinder its development. The high sequence variability of HCV genome, the lack of culture system for virus replication and small-animal models for HCV replication and pathogenesis are just some examples[10,14]. On top of these obstacles, intrinsic to HCV biology, there are a few difficulties of technical nature as the restrictions to use live attenuated or inactivated virus as vaccine candidates, the slow replication of HCV in culture systems which impact production yields, the lack of suitable protocols for large scale-production and purification and the natural instability of HCV during storage and transportation. As a result to all these limitations, virus-like particles emerge as optimal candidates to use as HCV vaccine[14].

In this work we have successfully produced HCV-Like particles as a result of ectopic expression of HCV proteins (Core, E1, E2, p7 and NS2) in HEK293 and HuH-7 cells. We started with the molecular cloning of several HCV genes in a plasmid used for the production of third generation lentiviral vectors able to infect a large variety of cells and tissues[41,64]. Five different HCV constructs were generated in the same vector backbone diverging in promoter and selection marker gene (Figure 4.1). Three of these constructs were used to generate lentiviral vectors which delivered HCV genes to target cells promoting a stable expression of the gene of interest. After transducing HEK293 and HuH-7 cells with the essential genes for HCV-LP production we evaluated gene expression by measuring mRNA (Figure 4.2A) and protein levels (Figure 4.2B). These results indicate that all viral components were expressed in both HEK293 and HuH-7 cells suggesting that both cells are producing HCV-LP. Even though variations in mRNA expression are visible in RT-qPCR data, we observed similar expression of each viral protein in Western blots. A possible explanation is the heterogeneity of cell populations and the increased susceptibility of RT-qPCR to small variations in RNA content.

Our efforts focus the continuous production of HCV-LP in mammalian cells, specifically HEK293 and HuH-7 cell lines. These cell lines were selected as candidate substrates for HCV-LP production because HuH-7, which derived from a human hepatoma, is a physiologically relevant culture model[65] for HCV, and HEK293 was selected because it is widely used in industrial processes being already validated in the biopharmaceutical industry and among regulatory agencies[66,67]. The use of simpler models for VLP production, as insect, yeast or bacterial cells is already reported in the literature[68]. The latter options presented important drawbacks as the low quality of HCV antigens (namely improper folding or post-translational modifications), low production yields of fully assembled virus particles, or the low immunogenicity of isolated viral antigens[26,68]. Therefore, we

next evaluate the productivity of transduced cell lines and the production of fully assembled viral particles.

Preliminary data indicate that a similar number of particles can be isolated from transduced HuH-7 and HEK293 cells supernatants, suggesting that both cells have similar productivities (data not shown). Even though these particles were underrepresented in non-transduced cells we failed to prove the presence of HCV antigens in these particles. A potential explanation is the low density of HCV antigens in these particles not reaching the lower limits of detection of our analytical tools (antibodies for western blot). We then hypothesize the presence of assembled viral particles entrapped in the intracellular space thus proceed with the purification of HCV-LP from intracellular extracts. HCV-LP were harvested from the intracellular space using physical freeze/thawing extraction protocols to avoid the potential negative impact of detergents to HCV-LP envelope membranes. The same extraction and purification protocols were applied to non-transduced cells and to cells transduced with HCV antigens except for the key structural *Core* gene (ΔC E1E2 p7NS2 sample). As visible in Figure 4.3 higher amounts of HCV envelope antigens are detected in samples where Core protein is present in higher amounts suggesting the intracellular production of fully assembled HCV-LP. An important step in industrial production of vaccine is the development of purification protocols based on chromatographic methods in opposition to ultracentrifugation protocols. We assessed the feasibility of using size-exclusion chromatography (SEC) to isolate HCV-LP from producer cells supernatant while evaluating the impact of FBS reduction in the production of HCV-LP. Despite the low concentration of HCV-LP in culture supernatants, these were successfully isolated by SEC (Figure 4.4). The condition resulting in higher purification yields was obtained for HuH-7 cells cultured in 5% FBS. This condition has the advantage of using half the concentration of FBS during HCV-LP production reducing the overall amount of contaminants which negatively impact the purification efforts.

The overall results show that both HuH-7 and HEK293 cell lines are suitable substrates for the production of HCV-LP with minimal differences identified. Therefore, we selected HEK293 cells to proceed for the development of a stable cell line for the continuous production of HCV-LP. This selection was based in the advantages that HEK293 cells present to the biopharmaceutical industry and regulatory agencies, with the accumulated know-how to manipulate HEK293 and with the variety of tools and protocols available to use with HEK293 cells.

To develop a cell-line suitable for industrial applications site-specific recombination systems are preferred over cumulative lentiviral transduction as the last poses some disadvantages. A major disadvantage is the random integration of the transgene in the target cells that can originate a highly heterogeneous cell population expressing from very low to very high amounts of the gene of interest. Moreover, this random integration can activate the expression of silent genes or silence the

expression of active genes with unpredictable consequences[69]. Also, the cumulative infection with lentiviral vectors can originate replication competent lentivirus due to uncontrolled recombination events, raising important safety concerns[51]. Therefore specific targeting of well-characterized genomic sites can help overcoming major problems downstream of cell-line development[51,70]. This strategy was successfully implemented for the production of therapeutic proteins and viral vectors in mammalian cells[50,51]. The success of this strategy depends on the efficient tagging of a genetic hotspot which is genetically stable, is easily targeted by the corresponding recombinase and the which supports high gene expression levels based in the integration of a single copy of the cassette[51].

For this part of the work we took advantage over the existence in the laboratory of a cell line previously tagged with a site-specific recombination cassette based on Cre-recombinase. We performed the construction of the tagging plasmid (Figure 4.5) which drives the expression of *mCherry* reporter gene to facilitate the identification of the desired genetic hotspots. The new tagging cassette was randomly integrated in HEK293 cell clones #1, #2 and #3 previously tagged with the *pTagLoxP_GFPZeo* cassette. The advantage of using fluorescence proteins[55] as reporter genes is the fast screen of positively tagged cells and to easily correlate this information with loci expression levels[61]. Coupling fluorescence reporter genes with antibiotic resistance genes allows not only the identification of mCherry positive cells but also the isolation of antibiotic resistant tagged cells from untagged cells which are sensitive to the presence of the antibiotic during cell culture.

Our data indicate that all cell clones HEK293 #1, #2 and #3 were successfully tagged with mCherry expressing cassette (Figure 4.6). Resistant hygromycin cells were generated in all transfected clones, curiously in every case a percentage of resistant cells did not present detectable mCherry fluorescence. A limiting dilution was performed to isolate mCherry positive from mCherry negative hygromycin resistant cells. After visual screening of individually growing cell aggregaty, thereby designed colonies, 16 were selected for expansion. After careful cell culturing of these 16 clones in hygromycin containing culture medium we evaluate the number of mCherry positive cells (Figure 4.7) and the intensity of mCherry and GFP reporter genes (Figure 4.8). Finally, 10 clones were selected to further proceed with cell characterization.

As mentioned, PEI induces rupture of cellular membrane which may lead to the delivery of multiple copies of its cargo. Since the success of the recombinase mediated cassette exchange methodology relies on the integration of a single copy of the tagging construct[51] it is thus mandatory to determine the number of copies present in the genome of tagged cells. When comparing our cell clones to a reference cell line known to have a single copy of *hygromycin* gene only 6 out of 10 clones (#103, #206, #301, #302, #306 and #311) were validated for a single copy (Figure 4.9). This approach was described by Bodin *et al in* 2005[56] and it correlates the relative amount of DNA with copy

numbers of gene of interest[71]. Clones are considered single copy when relative values are below 1.4 times the relative control[56].

In addition to FRT recombination sites, a functional region present in *pTagFRT_mCherry* tagging cassette is the retrovirus packaging signal (Ψ) thus mCherry coding proviral RNA can be incorporated in MLV particles[63]. Therefore, our last characterization of integrated cassette evaluated the delivery of mCherry coding RNA to non-fluorescence HEK293 cells by MLV viral vectors produced in HEK293 #103, #206, #301 and #311 tagged cell clones. As visible in Figure 4.10, clones #206, #301 and #311 were able to generate infectious particles reflecting the successful encapsidation of mCherry coding mRNA. We proceed for site specific recombination in clones #206 and #311.

Clones #206 and #311 were thus co-transfected with *pTarLoxP_E1E2* and Cre-recombinase expression vectors using either PEI or $CaPO_4$. These are currently the two most cost-effective and efficient transfection vehicles used in large-scale transfection of mammalian cells[72]. *pTarLoxP_E1E2* target cassette encodes both HCV envelope glycoproteins, E1 and E2, and contains an internal promoter that will drive the expression of the promoterless *puromycin* gene after successful recombination with the tagged cassette. This system assures selection of recombinant cells by transcription initiation of *puromycin* resistance gene. In the absence of this positive selection with puromycin, random integration would be favored over RMCE since recombination is only possible when a series of factor converge in one genomic site whereas random integration can occur widespread in the whole genome[61]. An average of 7% cassette exchange efficiency was determined for both PEI and $CaPO_4$ transfection by flow cytometry (Figure 4.11). Similar to tagged clones isolation, limiting dilution of recombinant cell populations allowed the isolation of a single recombinant GFP negative and puromycin resistant cell clone named #311_F8 (Figure 4.12) which was expanded and cryopreserved for further use.

In the end, parental clone #311 and clone #311_F8 were in parallel co-transfected with *pTarFRT_Core* and *pSVFlpe* plasmids to target mCherry expression locus with HCV *Core* gene allowing production of HCV-LP. Successful recombination of *pTarFRT_Core* with *pTagFRT_mCherry* mediated by flipase enzyme induces the expression of a defective *neomycin* resistance gene by restoring the ATG starting codon which is lacking upstream the open reading frame. Neomycin selection and clone expansion of resistant cells is still ongoing and it will encourage the continuation of this work since it will significantly contribute for future work.

The huge potential of VLPs for biotechnological and pharmaceutical applications renders this master project not only scientifically but also technologically relevant. Moreover, RMCE systems constitute an excellent tool for a VLPs production platform. Thus, this project contributes greatly to the advancement of HCV-LPs production and purification for HCV vaccine.

6. Conclusion and Future Work Perspectives

The main objective of this work was to generate HCV-like particles and the development of a production platform compatible with industrial production requirements.

This work started by evaluating the production of HCV-LP in different cell substrates (HEK293 and HuH-7) after which we selected the best performing candidate for cell line development using defined cell engineering tools and protocols.

Our data suggests that HCV-LP produced in HEK293 cells are of similar quality to those produced in physiologically compatible HuH-7 cells with the advantage of being suitable for industrial applications. Therefore, HEK293 cells were used as substrates for cell line development using site-specific recombination tools which allows cell line development independent from viral-vectors.

With this work we were able to develop two cell lines harboring continuous production of fully assembled HCV-LP. Different protocols for HCV-LP purification, including size-exclusion chromatography, were tested and which can be easily adapted to large-scale processes as required by the biopharmaceutical industry. Moreover, we have developed and characterized a new cell-line with two highly flexible site-specific recombination systems for the development of HCV-LP from defined high expression loci. The potential of the applications of this new cell line go far beyond the aims of this thesis.

At the moment, a variety of specific aspects of vaccine production are being optimized in the laboratory of Cell Line Development and Molecular Biotechnology. This includes (i) improving HCV-LP production by enhancing the expression of HCV structural genes and co-expression of non-structural enzymatic genes, (ii) improving HCV-LP purification protocols by testing different harvesting and purification protocols and (iii) the development of a protocol to evaluate the functionality of HCV-LP displayed antigens to assess quality and stability of production batches.

In the future, the immunogenicity of HCV-LP produced in different cell substrates should be evaluated in animal models to better sustain the decision over which is the best platform for vaccine production. Moreover, HCV-LP produced from cells expressing only structural (Core, E1, E2) genes or structural and non-structural (p7, NS2, NS3, NS4a, NS4b and NS5a) genes should also be evaluated since the last can play a role in viral particles productivity and/or antigen display and maturation thus contributing for improved vaccine production yields and performance.

7. References

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8. Attachments

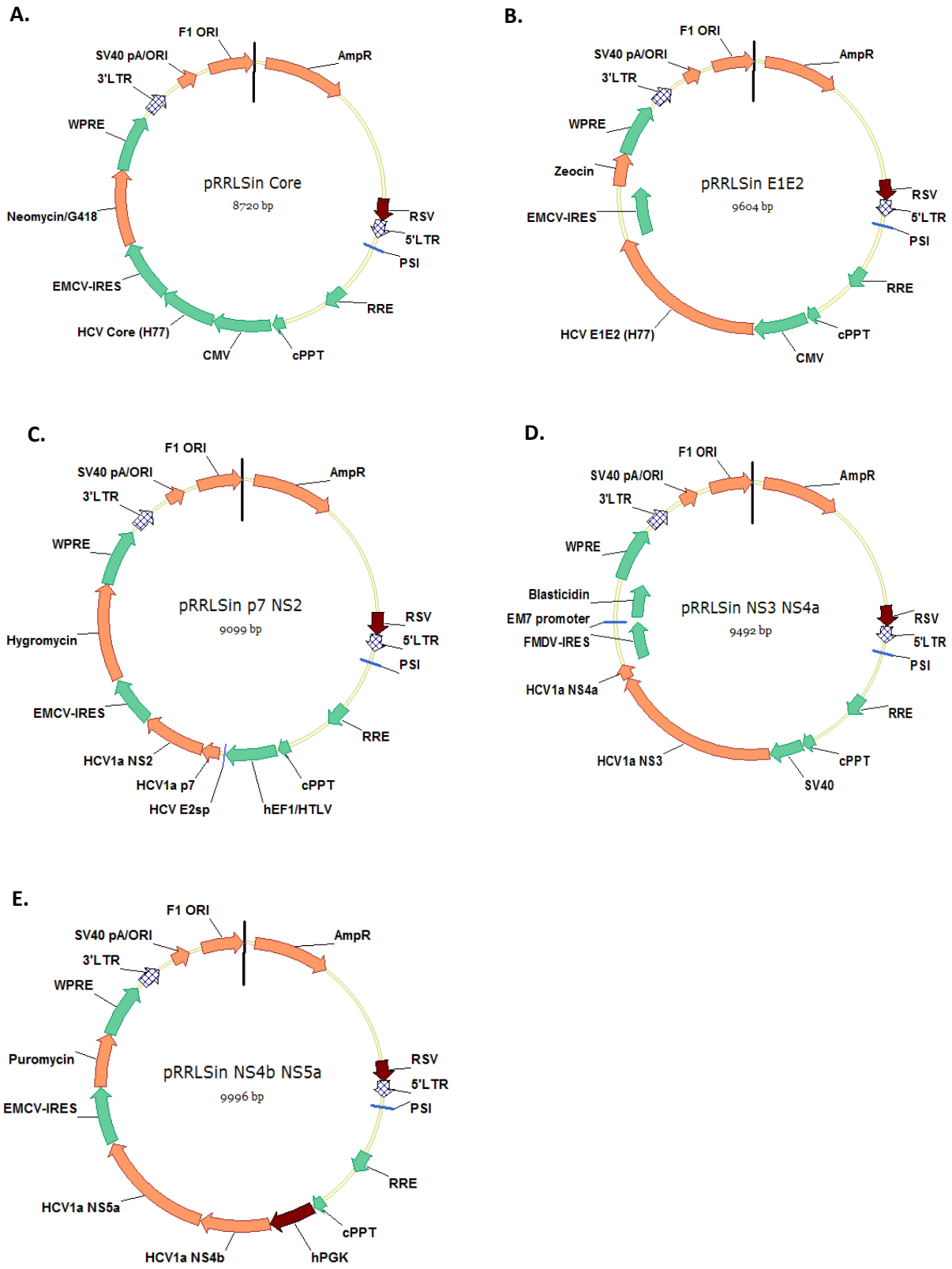

Figure 8.1. Final lentiviral plasmid constructs. A-*pRRLSin_Core*; B-*pRRLSin_E1E2*; C-*pRRLSin_p7_NS2;* D-*pRRLSin_NS3_NS4a;* E-*pRRLSin_NS4b_NS5a*

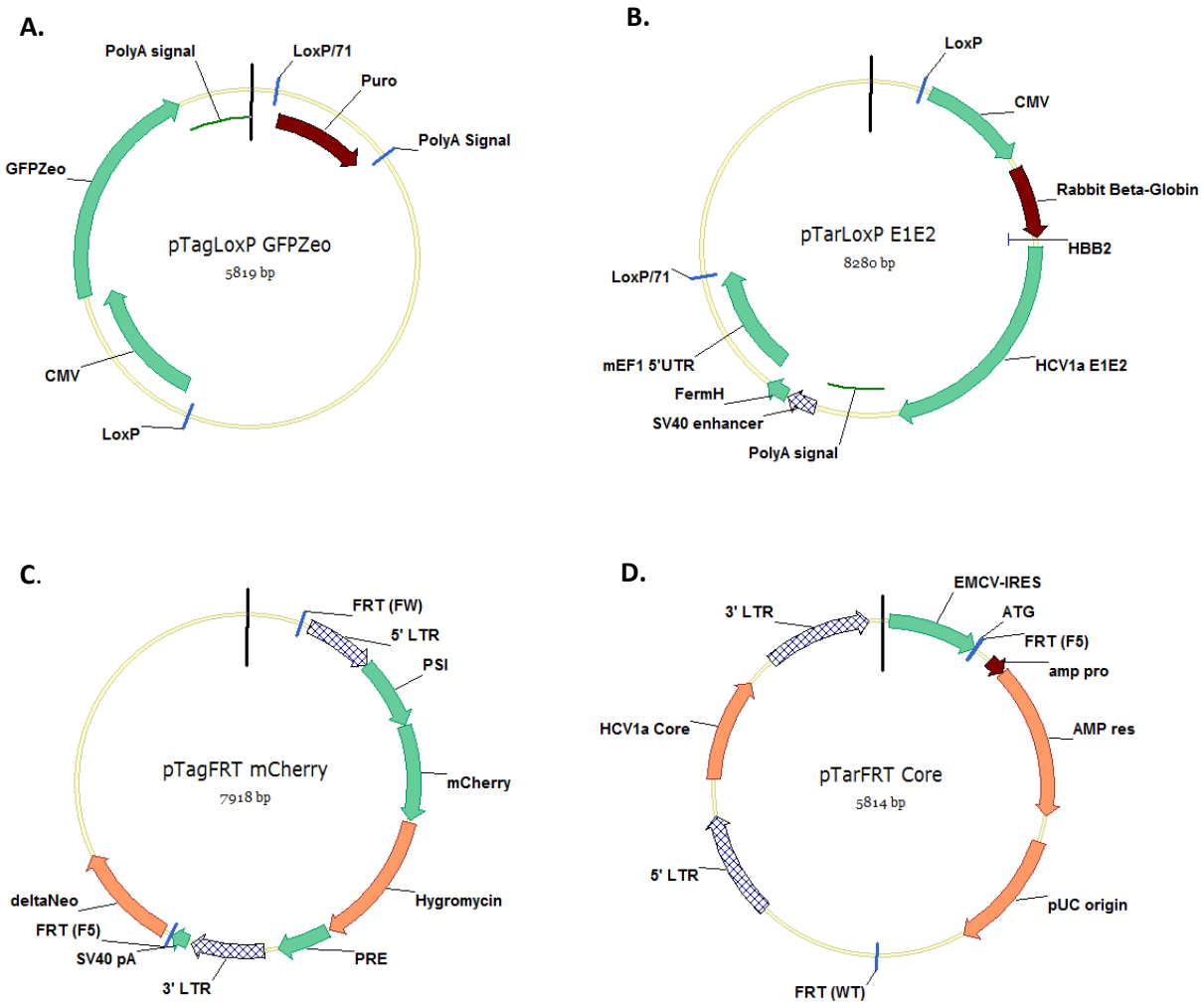

Figure 8.2. Final cassette exchange plasmid constructs. A-*pTagLoxP_GFPZeo* (tagging plasmid); B-*pTarLoxP_E1E2* (targeting plasmid); C-*pTagFRT_mCherry* (tagging plasmid) and D-*pTarFRT_Core* (targeting plasmid).

Table I. List of primers and templates used for construction of lentiviral transgenes. A-*pRRLSin_Core*; B-*pRRLSin_E1E2*; C-*pRRLSin_p7_NS2;* D-*pRRLSin_NS3_NS4a;* E-*pRRLSin_NS4b_NS5a.*

A.

Insert	Template	Primers	Plasmid backbone	Restriction enzymes or primers	
Neo	*pEMC_Galeo*	Fw - GAAAAACACGATGATATACCGGTATGAGTGGATTGCACGCAGG Rev - CAGAGGTTGATTGTCGACTTACTATCAGAAGAACTCGTCAAG	*pRRLSin_GFP_S10*	Age I and Sal I	*pRRLSin_GFP_S10_Neo (1)*
Core	*pGEM_Core*	Fw - CGTCAGATCCGCTAGCATGAGCACGAATCCTAAACCTCAA Rev - TTTCGCGGGGGATCCTCATTACTAGGCTGAAGCGGGCAC	*pRRLSin_GFP_S10_Neo (1)*	Bam HI and Nhe I	***pRRLSin_CMV_Core_IRES_ Neo***

B.

Insert	Template	Primers	Plasmid backbone	Restriction enzymes or primers	
E1E2	*pEPX145-71*	Fw - CGTCAGATCCGCTAGCGATGGCGGCGGATTTGATGGGCTA Rev - TAATTTCGCGGGGGATCCAGCCTCGGCTTGGCTAATGA	*pRRLSin_GFP_S10*	Bam HI and Nhe I	***pRRLSin_CMV_E1E2_IRES_ Zeo***

C.

Insert	Template	Primers	Plasmid backbone	Restriction enzymes or primers	
hEF1/HTLV	*pPuro_mCherry*	Fw - GGCCCGACGTCGCATGCTCGAGGATCTGCGATCGCTCCGGTGCCCG Rev - GACCTGCAGGCGGCCGCGTAGGCGCCGGTCACAGCTTGGATC	*pGEM_Core*	Not I and Sph I	*pGEM_hEF1/HTLV (1)*
p7*	*pGEM_p7*	Fw - GTTACTCATATCCCAAGCGGAGGCGGCTTTGGAGAACCTCGTAATACTCA Rev - CCCATATGGTCGACCATGGTGCGTATGCCCGCTGAGGCAACGCC	———	———	*p7 (1)*
	p7 (1)	Fw - TGCTCCTGCTTGTGGATGATGTTACTCATATCCCAAGCGGAGGCG Rev - CCCATATGGTCGACCATGGTGCGTATGCCCGCTGAGGCAACGCC	———	———	*p7 (2)*
	p7 (2)	Fw - ACCGGTACCATGCGCGTCTGCTCCTGCTTGTGGATGAT Rev - CCCATATGGTCGACCATGGTGCGTATGCCCGCTGAGGCAACGCC	———	———	*p7 (3)*
	p7 (3)	Fw - GACCGGCGCCTACGCACCGGTACCATGCGCGTC Rev - CCCATATGGTCGACCATGGTGCGTATGCCCGCTGAGGCAACGCC	*pGEM_hEF1/HTLV (1)*	Not I and Pst I	*pGEM_hEF1/HTLV_p7 (2)*
NS2	*pGEM_NS2/3*	Fw - AGCGGGCATACGCACTGGACACGGAGGTGGCCGCGTCGTG Rev - GGGAGCTCTCCCATATTACAGCAACCTCCACCCCTTGGAGACC	*pGEM_hEF1/HTLV_p7 (2)*	Nco I and Nde I	*pGEM_hEF1/HTLV_p7_ NS2 (3)*
IRES_Hygro	*pRRLSin_UNUM_ IRES_Hygro*	Fw - GTAATATGGGAGAGCTCGTTATTTTCCACCATATTGCCGTCT Rev - ATCCAACGCGTTGGGGTCGACCTATTCCTTTGCCCTCGGACGAGTG	*pGEM_hEF1/HTLV_p7_ NS2 (3)*	Sac I	*pGEM_hEF1/HTLV_p7_NS2_ IRES_Hygro (4)*
hEF1/HTLV_p7_ NS2_IRES_Hygro	*pGEM_hEF1/HTLV_p7_NS2_ IRES_Hygro (4)*	Fw - GATCACGAGACTAGCCTCGAGGATCTGCGATCGCTC Rev - GAGGTTGATTGTCGACCTATTCCTTTGCCCTCGGAC	*pRRLSin_GFP_S10_Neo*	Sal I and Xho I	***pRRLSin_hEF1/HTLV_p7_ NS2_IRES_Hygro***

*Note: p7 fragment was amplified using "Assembly PCR" strategy to introduce 54 amino acids signal peptide at N-terminus.

Table I. List of primers and templates used for construction of lentiviral transgenes. A-*pRRLSin_Core*; B-*pRRLSin_E1E2*; C-*pRRLSin_p7_NS2;* D-*pRRLSin_NS3_NS4a;* E-*pRRLSin_NS4b_NS5a* (continuation).

D.

Insert	Template	Primers	Plasmid backbone	Restriction enzymes or primers	
SV40	*pZeoCre*	Fw - GCCCGACGTCGCATGCTGTGGAATGTGTGTCAGTT Rev - GACCTGCAGGCGGCCCGAAAATGGATATACAAGCT	*pGEM_Core*	Not I and Sph I	*pGEM_SV40 (1)*
NS3	*pGEM_NS2/3*	Fw - TTCGGGCCGCCTGCATGGCGCCCATCACGGCGTACGC Rev - GGGAGCTCTCCCATACGTGACGACCTCCAGGTCGG	*pGEM_SV40 (1)*	Nde I and Pst I	*pGEM_SV40_NS3 (2)*
NS4a	*pGEM_NS4a*	Fw - GTCGGCCGACCTGGAAGCACCTGGGTGCTCGTTGG Rev - GCATCCAACGCGTTGTTAGCACTCTTCCATCTCATCGA	*pGEM_SV40_NS3 (2)*	Fw - CGTGACGACCTCCAGGTCGGCCGACATGCATG Rev - CAACGCGTTGGATGCATAGCTTGAGTATTC	*pGEM_SV40_NS3/4a (3)*
IRES_Blast	*pREV_IRES_Blast*	Fw - GAAGAGTGCTAACAAAGCAGGTTTCCCCAATGACAC Rev - CCTTTTGCTCACATGTTAGTTCCTGGTGTACTTGAG	*pGEM_SV40_NS3/4a (3)*	Fw - CATGTGAGCAAAAGGCCAGCAAAAGG Rev - TTGTTAGCACTCTTCCATCTCATCG	*pGEM_SV40_NS3/4a_ IRES_Blast (4)*
SV40_NS3/4a_ IRES_Blast	*pGEM_SV40_NS3/4a_ IRES_Blast (4)*	Fw - GATCACGAGACTAGCCCTGTGGAATGTGTGTCAGTT Rev - GAGGTTGATTGTCGATTAGTTCCTGGTGTACTTGAG	*pRRLSin_GFP_S10_Neo*	Sal I and Xho I	***pRRLSin_HCV_SV40_NS3/4a_ IRES_Blast***

E.

Insert	Template	Primers	Plasmid backbone	Restriction enzymes or primers	
hPGK	*pRRLSin_hPGK*	Fw - GCCCGACGTCGCATGCCACGGGGTTGGGGTTGCGC Rev - GACCTGCAGGCGGCCGCCCTGGGGAGAGAGGTCGGTG	*pGEM_Core*	Not I and Sph I	*pGEM_hPGK (1)*
NS4b	*pGEM_NS4b*	Fw - CTCTCTCCCCAGGGCATGTCTCAGCACTTACCGTACAT Rev - CCCATATGGTCGACCGGAGCATGGAGTGGTACACTCCG	*pGEM_hPGK (1)*	Not I and Pst I	*pGEM_hPGK_NS4b (2)*
NS5a	*pGEM_NS5a*	Fw - CACTCCATGCTCCGGTTCCTGGCTAAGGGACATCT Rev - GGGAGCTCTCCCATAttaGCAGCACACGACATCTTCCG	*pGEM_hPGK_NS4b (2)*	Nde I and Sal I	*pGEM_hPGK_NS4b/5a (3)*
IRES_Puro	*pRRLSin_IRES_Puro*	Fw - CTAATATGGGAGAGCTCCCAACCCGCGAAATTAATACGACTC Rev - CTATGCATCCAACGCGTTAAGCTCCAGGCTTCCTTG	*pGEM_hPGK_NS4b/5a (3)*	Mlu I	*pGEM_hPGK_NS4b/5a_ IRES_Puro (4)*
hPGK_NS4b/5a_ IRES_Puro	*pGEM_hPGK_NS4b/5a_ IRES_Puro (4)*	Fw - GATCACGAGACTAGCCCCACGGGGTTGGGGTTGCGC Rev - GAGGTTGATTGTCGATTAAGCTCCAGGCTTCCTTG	*pRRLSin_GFP_S10_Neo*	Sal I and Xho I	***pRRLSin_HCV_hPGK_NS4b/5a_ IRES_Puro***

Table II. List of primers and templates used for construction of plasmids for cassette exchange.

Insert	Template	Primers	Plasmid backbone	Restriction enzymes	Final constructions
GFPZeo	*pSELECT-GFPZeo-LacZ*	Fw - CTGAATGCGTAAAATACAGCATAGCAAAACTT Rev - TAGATATCGCTCACATGTTCTTAATTAACCT	*pTagLoxP-mcs*	Bsm I and Eco RV	***pTagLoxP_GFPZeo***
E1E2	*pEPX145-71*	Fw - GGCTCTTAATTAACATTACCGCCATGTTGACATT Rev - TAACGCCTAGGAGTGTGCTGGAATTGCC	*pTarLoxP-mcs*	Avr II and Pac I	***pTarLoxP_E1E2***
mCherry	*pRSV-CherryPuro*	Fw - CTTACAGGCGCGGCCGCATGGTGAGCAAGGGCGAGGAG Rev - GAGATCTTTAATTAATTACTTGTACAGCTCGTCC	*pUC1S_UNUM113*	Not I and Pac I	***pTagFRT_mCherry***
Core	*pGEM_Core*	Fw - GACGAGTTCGGAACACCCATGAGCACGAATCCTAAACC Rev - GTTCTAGAGTCGCGGCCTTATTAGGCTGAAGCGGGCAC	*pEmMFG*	Eag I	***pTarFRT_Core***

Table III. List of primers used for RT-qPCR.

RPL-22	Fw - CTGCCAATTTTGAGCAGTTT
	Rev - CTTTGCTGTTAGCAACTACGC
Hygro	Fw - CAAAGATCGTTATGTTTATC
	Rev - GTAGTGTATTGACCGATTCC
Core	Fw - GCATGAGCACGAATCCTAAA
	Rev - CGGCAACAAGTAAACTCCAC
E1	Fw - GGCCAGCTGTTTACCTTCTC
	Rev - ATCATCATATCCCAGGCCAT
E2	Fw - GTGGTGGTTGGCACTACAGA
	Rev - TCCAAGTACACCCAAACCAA
p7	Fw - CACGGTCTTGTGTCCTTCC
	Rev - AGCAGGAGCAGGAGGAGAG
NS2	Fw - CGATGCCGTCATCTTACTCA
	Rev - AACTGGCTTGAAGAATCCAAA